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Cellular and molecular mechanisms of immunoregulatory action of stem cells and their effect on adaptive immune cells

Buněčné a molekulární mechanismy imunoregulačního působení kmenových buněk a jejich vliv na buňky adaptivní imunity

DOCTORAL THESIS

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Abstract

Regulation of immune reactions represents an entire system of maintenance of homeostasis, self-tolerance, and host defense. Regardless of intensive research, the cellular and molecular insights into immunomodulation remain incomplete. Therefore, we aimed to study different approaches to modulate the immune system, primarily focused on the induction, expansion, and activation of immunoregulatory cells.

We analyzed the therapeutic effect of the combined action of mesenchymal stem/stromal cells (MSCs) and immunosuppressive drugs on the balance among T cell populations. We found that MSCs ameliorated unfavorable effects of immunosuppressants on T cell activation. As a result of this approach, T cell development was altered from the T helper (Th) 1, Th2, and Th17 cell polarization to anti-inflammatory regulatory T cell-mediated response. Additionally, we studied the effect of the immunoregulatory action of MSCs on B cells. We evaluated the impact of cytokine-primed MSCs on the induction of interleukin (IL)-10-producing B cells. Results revealed that interferon (IFN)-γ- and IL-4-primed MSCs suppressed the production of IL-10 by activated B cells. This suppression was dependent on cell-to-cell contact. In the case of IFN-γ-primed MSCs, the inhibition of IL-10 secretion involved the cyclooxygenase-2 signaling pathway, but the suppression mediated by IL-4-primed MSCs was independent of this enzyme and its products.

Further, we investigated the role of the cytokine milieu in the development of IL-10-producing B and T cells. Moreover, we analyzed the involvement of GATA-3 and FoxP3 transcription factors in IL-10 production by activated B cells. IFN-γ significantly enhanced the proportion of IL-10-producing B cells, but IL-4 and transforming growth factor (TGF)-β decreased the percentage of these cells. The IL-10 expression in stimulated B cells was independent of GATA-3 and FoxP3 expression, contrasting with findings in T cells, where IL-10 expression was associated with GATA-3 or FoxP3 transcription factor after IL-4 or TGF-β stimulation. Simultaneously, we found that stimulated B cells, in comparison to stimulated T cells, nonspecifically bound R-phycoerythrin-conjugated antibodies during intracellular marker staining. Thus, the data acquired using these antibodies for intracellular staining of activated B cells must be taken with precaution.

The results presented in this thesis highlight the importance of regulation of immune response and its targeted modulation that can provide advanced therapeutic strategies for the treatment of autoimmune diseases or achievement of transplantation tolerance. Furthermore, the data stress the pivotal role of the cross-talk between cells and the local environment in the induction, expansion, and activation of immunoregulatory cells.

Abstrakt

Regulace imunitní odpovědi představuje komplexní systém, který slouží k udržení homeostázy, imunologické tolerance a obrany proti patogenům. Znalosti týkající se regulace imunitních reakcí však navzdory intenzivnímu výzkumu zůstávají omezené a nedostatečné. Proto jsme se zaměřili na studium různých možností indukce, expanze a aktivace immunoregulačních buněk.

První studie sledovala na spolupůsobení mesenchymálních kmenových/stromálních buněk (MSC) a imunosupresivních léků na podíl jednotlivých populací T lymfocytů. Zjistili jsme, že nežádoucí účinky imunosupresiv na aktivaci T lymfocytů byly pomocí MSC sníženy a došlo ke změně poměru mezi jednotlivými populacemi T lymfocytů během jejich vývoje. Byl utlumen rozvoj pomocných T (Th) lymfocytů typu 1, Th2 a Th17 lymfocytů, naopak došlo k podpoře vývoje protizánětlivých regulačních T lymfocytů. Následně jsme se zabývali imunomodulačními vlastnostmi MSC a jejich vlivem na B lymfocyty se zaměřením na efekt MSC ovlivněných cytokiny na B lymfocyty produkující interleukin (IL)-10. Výsledky ukázaly, že MSC, které byly ovlivněny interferonem (IFN)-γ a IL-4, signifikantně potlačovaly produkci IL-10 pomocí aktivovaných B lymfocytů, přičemž tota suprese byla závislá na přímém kontaktu mezi buňkami. V případě MSC ovlivněných IFN-γ byla inhibice sekrece IL-10 B lymfocyty závislá signální dráze cyklooxygenázy 2, ale potlačení způsobené MSC ovlivněnými IL-4 bylo nezávislé na její aktivitě a produktech.

Následně jsme studovali účinky cytokinového prostředí na vývoj B a T lymfocytů produkujících IL-10, přičemž jsme analyzovali roli GATA-3 and FoxP3 transkripčních faktorů na tuto produkci. Procento B lymfocytů produkujících IL-10 bylo signifikantně zvýšeno pomocí IFN-γ, ale IL-4 a transformující růstový faktor (TGF)-β toto zastoupení snižovaly. Kromě toho exprese IL-10 v stimulovaných B lymfocytech byla nezávislá na expresi transkripčních faktorů GATA-3 a FoxP3. Naopak u T lymfocytů exprese IL-10 byla asocionána s expresí GATA-3 a FoxP3 po stimulaci cytokiny IL-4 a TGF-β. Zároveň jsme ukázali, že aktivované B buňky nespecificky vážou protilátky konjugované s R-fykoerythrinem, pokud jsou použity pro značení intracelulárních molekul. Z toho důvodu by výsledky získané použitím těchto protilátek pro intracelulární znečení měly být brány s obezřetností.

Výsledky předložené v rámci této práci zdůraznily význam regulace imunitní odpovědi a její cílené modulace, která může poskytnout pokročilé terapeutické strategie pro léčbu autoimunitních onemocnění nebo dosažení transplantační tolerance. A rovněž potvrdily klíčovou roli komunikace mezi buňkami imunitního systému a lokálním prostředím a jejím vlivem na indukci, expanzi a aktivaci imunoregulačních buněk.

Content

List of abbreviations

APC	allophycocyanin
APCs	antigen-presenting cells
ATP	adenosine triphosphate
BCR	B cell receptor
Bregs	regulatory B cells
CD	cluster of differentiation
CIA	collagen-induced arthritis
ConA	concanavalin A
COX-2	cyclooxygenase-2
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCR7	C-X-C motif chemokine receptor
DCs	dendritic cells
EAE	experimental autoimmune encephalitis
FasL	Fas ligand
FITC	fluorescein isothiocyanate
FO	follicular
FoxP3	forkhead box P3
GATA-3	GATA binding protein 3
GITR	glucocorticoid-induced tumor necrosis factor receptor
GvHD	graft versus host disease
HIF-1α	hypoxia-inducible factor-1α
HLA	human leukocyte antigen
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
LAG-3	lymphocyte activation gene 3
LPS	lipopolysaccharide
MHCII	major histocompatibility complex class II
Mregs	regulatory macrophages
MS	multiple sclerosis
MSCs	mesenchymal stem/stromal cells

MZ	marginal zone
NK	natural killer
PD-L	programmed death-ligand
PGE_2	prostaglandin E2
RA	rheumatoid arthritis
RORγt	retinoic-acid-receptor-related orphan nuclear receptor γ
R-PE	R-phycoerythrin
SDF-1	stromal cell-derived factor-1
SLE	systemic lupus erythematosus
T1D	type I diabetes
T-bet	T-box transcription factor
TCR	T cell receptor
tDCs	tolerogenic DCs
TGF-β	transforming growth factor-β
Th	T helper
TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TLR	toll-like receptors
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Tregs	regulatory T cells

1. Overview of literature

1.1. Introduction

Regulation of immune reactions represents a crucial mechanism of balance between maintenance body physiology and host defense. Specific and effective launch of immune response is the primary requirement for sufficient pathogen clearance. Nevertheless, this response needs to burn out quickly to avoid adverse tissue damage. Immune overreactions are the initial source of disbalance, leading to the development of autoimmune diseases.

Immunoregulatory cells are involved in the maintenance of homeostasis and self-tolerance. These cells contribute to the preservation of tolerogenic orchestra. They can develop from different cell lines and give rise to regulatory or tolerogenic phenotypes of targeted cells. The expansion and recruitment of regulatory cells appear as a promising tool for cell-based therapy of autoimmunity and immune reaction against allograft, achieving specific and personalized treatment avoiding side effects of systemic drug administration.

Despite intensive research, important questions concerning the induction and development of immunoregulatory cells remain unclear and unanswered. For these reasons, we analyzed the conditions for the induction and activation of different regulatory cells and their mutual interactions. The deep insight into immune regulation is a golden grail in treating autoimmune disorders and achieving transplantation tolerance.

1.2. Immunoregulatory cells

Regulation of immune responses is the basis of tolerance, leading to maintain homeostasis and self-tolerance of living systems. Generally, the tolerance can be divided into central and peripheral tolerance. Central tolerance is based on the elimination of autoreactive T and B cells during their development in the thymus or bone marrow. The primary mechanism is clonal deletion when self-reactive cells are eradicated by apoptosis (Xing and Hogquist, 2012).

On the other hand, peripheral tolerance involves the elimination and prevention of potentially dangerous immune responses against self-tissues or harmless antigens, such as food or commensal organisms, in peripheral organs (Mueller, 2010). Regulatory T cells (Tregs) have been initially considered to perform immunoregulation (Hsieh et al., 2012). However, peripheral tolerance is based on many different cell types. These immunoregulatory cells develop in the periphery under a specific microenvironment (Buckner and Ziegler, 2004; Luan et al., 2014).

Immune responses work as a system of positive or negative feedback loops, which can promote and boost immune reactions to eliminate the danger and, at the same time, let these reactions burn out and prevent the response against self- and harmless antigens. Their regulation includes an extensive range of feedback loops on all levels of the immune system. The immunoregulatory cells represent an important level of regulation that participates in maintaining homeostasis.

1.2.1. Regulatory cells of innate immunity

The innate immune system performs the first line of protection against pathogens involving physical, chemical, and cellular defenses. It is based on immediate recognition of dangerous signals and prompt reaction. This is mediated by pathogen-recognition receptors that bind the molecules containing pathogen-associated molecular patterns, representing a restricted set of evolutionary-conserved molecular structures carried by various pathogens (Buchmann, 2014).

Besides the first line of pathogen defense, the specific innate immune cells can also provide the connection between innate and adaptive immune systems. The association is provided by antigen-presenting cells (APCs), including especially dendritic cells (DCs) and macrophages. APCs are critical players in the initiation of immune response (Gaudino and Kumar, 2019). Both DCs and macrophages are heterogeneous groups of cells that can be divided into different subpopulations. They can also acquire immunoregulatory functions. Subpopulations that participate in the regulation of immune responses involve tolerogenic DCs (tDCs) and regulatory macrophages (Mregs) (Chandrasekaran et al., 2019; Iberg and Hawiger, 2020).

1.2.1.1. Tolerogenic dendritic cells

tDCs contribute to the maintenance of homeostasis and tolerance by deletion of T cells recognizing self-antigens, by anergy of T cells, the suppression of effector T cell responses, and the induction of Tregs (Domogalla et al., 2017). Although the immunoregulatory tDC phenotype was originally associated with immature DCs, which exhibit the low expression of co-stimulatory molecules (cluster of differentiation (CD)80 and CD86) and the major histocompatibility complex class II (MHCII), it has been shown that DCs expressing mature phenotype can also induce Tregs (Kim and Kim, 2019; McGuirk et al., 2002; Menges et al., 2002). These semi-mature DCs, so-called steady-state migratory DCs, exert the expression of

high levels of MHCII and co-stimulatory molecules but can still induce antigen-specific interleukin (IL)-10-producing Tregs (Lutz and Schuler, 2002).

tDCs can induce tolerance via different mechanisms, involving several pathways and targeting different immune cell types. Among these mechanisms belong clonal anergy and deletion of T cells, metabolic modulation of effector cells, and anti-inflammatory cytokine production (Morante-Palacios et al., 2021). Clonal anergy can be triggered when T cell receptor (TCR) binds to MHCII molecule expressed on tDCs in the absence of proper co-stimulatory signals, leading to T cell proliferation reduction (Fathman and Lineberry, 2007). Moreover, T cell anergy can also occur in the presence of co-inhibitory molecules on tDCs. Distinct subpopulations of tDCs express programmed death-ligand (PD-L) 1 and 2 (Sage et al., 2018; Vander Lugt et al., 2017).

In addition to cell-to-cell contact-dependent mechanisms, tDCs produce different anti-inflammatory cytokines, such as IL-10 or transforming growth factor-β (TGF-β), which trigger the induction and differentiation of suppressive Tregs (Awasthi et al., 2007; Boks et al., 2012). Furthermore, tDCs can express indoleamine 2,3-dioxygenase (IDO), which arrests the cell cycle of T cells via tryptophan depletion (Mellor et al., 2017). tDCs can also reduce T cell proliferation through lactate secretion (Marin et al., 2019).

Finally, tDCs can achieve the elimination of T cells via deletion. The promotion of T cell apoptosis is triggered directly through the expression of death receptor ligands on tDCs, such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) (Fanger et al., 1999; Li and Shi, 2015).

tDCs participate in the maintenance of homeostasis through the suppression of autoimmune reactions. tDCs can reprogram pathological inflammatory and autoimmune reactions, which can be considered as tDC-based therapy. The curative potential of tDCs have been studied in clinical trials concerning the treatment of graft versus host disease (GvHD) in transplantation and autoimmune diseases involving multiple sclerosis (MS), type I diabetes (T1D), or rheumatoid arthritis (RA) (Bell et al., 2017; Moreau et al., 2012; Phillips et al., 2019; Willekens et al., 2019).

1.2.1.2. Regulatory macrophages

Mregs represent one of three primary macrophage subpopulations. Firstly, classically activated M1 macrophages are effector macrophages that produce high levels of pro-inflammatory cytokines potentiating inflammatory reactions. Secondly, wound healing M2 macrophages can contribute to the clearance of helminths and nematodes. Nevertheless,

their primary function is to restore and remodel the extracellular matrix after tissue injury. Thirdly, Mregs have anti-inflammatory and immunosuppressive properties through which they regulate homeostasis. Mregs are characterized by the production of large amounts of anti-inflammatory IL-10 and TGF-β cytokines and lack production of IL-12 (Mosser and Edwards, 2008).

Although M2 macrophages and Mregs share certain immunoregulatory properties, they can be distinguished based on antigen-specific responses. Unlike M2 macrophages, Mregs express substantial levels of CD80 or CD86 co-stimulatory molecules and, therefore, can present antigens to T cells. On the other hand, Mregs are unable to express a high level of arginase, which is characteristic of M2 macrophages (Zhang et al., 2021). Furthermore, Mregs suppress the activation of M2 macrophages and limit the pathological role of M2 macrophages in fibrosis (Chandrasekaran et al., 2019).

Besides, Mregs attenuate the proliferation of mitogen-stimulated T cells and reduce the proportion of cocultured T cells by phagocytosis in the allospecific manner (Riquelme et al., 2013). Consistent with the immunoregulatory function of Mregs, they further promote differentiation of Tregs, thereby increasing the Treg number in recipient blood after kidney transplantation (Riquelme et al., 2018).

1.2.2. Regulatory cells of adaptive immunity

The adaptive immune system is evolutionarily younger than the innate immune system. The adaptive immune system is antigen-specific and antigen-dependent. It is centered on lymphocytes, B and T cells, carrying antigen-specific receptors, B cell receptors (BCR) and TCR. Both types of antigen-specific receptors are generated by somatic mutations, which create an incredibly diverse and highly specific repertoire recognizing epitopes. Adaptive immunity develops immunological memory, providing rapid and efficient immune response upon the subsequent antigen exposure. Because of the power of adaptive immune reactions, they are regulated by innate immune cells, resulting in synergy between them. Moreover, the adaptive immune system depends on lineage-specific regulatory cells, which help balance pro- and anti-inflammatory reactions (Flajnik and Kasahara, 2010; Marshall et al., 2018).

As it has been mentioned above, the adaptive immune system consists of only two different types of cells, B and T cells. Nevertheless, these lineage-specific cell types are further divided into many different subpopulations, among which regulatory B cells (Bregs) and Tregs belong. Tregs were described more than 10 years before Bregs (Hall et al., 1990; Mizoguchi et

al., 2002). However, both Bregs and Tregs play a pivotal role in the modulation of immune system reactions by participating in the balance and the maintenance of homeostasis and self-tolerance.

1.2.2.1. Regulatory T cells

Tregs are the specialized immunoregulatory population of $CD4^+$ T cells, which can attenuate extensive and uncontrolled immune reactions involving both adaptive and innate parts of the immune system. Tregs play a central role in tolerance are a fundamental basis of immune self-tolerance and maintenance of homeostasis (Bayati et al., 2021).

Many studies have shown that Tregs can ameliorate graft rejection in transplantations; hence their administration can potentially minimalize the usage of immunosuppressive drugs (Trzonkowski et al., 2009). Tregs also attenuate the disorder and damage progression in autoimmune diseases. On the other hand, immunosuppressive conditions based on Treg action might help tumorigenic growth to escape immune surveillance and result in cancer (Bayati et al., 2021).

Origin, phenotype, and markers

Tregs were initially described as $CD4^+CD25^+$ T cells with anergic and suppressive functions in mice (Sakaguchi et al., 1995). Later, it was shown that $CD4^+CD25^+$ Tregs express forkhead box P3 (FoxP3) transcription factor as their master regulator and defining marker. Furthermore, they represent specific thymus-derived T cell lineage (Fontenot et al., 2003; Itoh et al., 1999; Ng et al., 2001).

Two different subpopulations of Tregs can be distinguished depending on their development. Natural Tregs develop in the thymus by positive selection when self-reactive TCR on $CD4^+$ thymocytes recognize self-peptides in MHCII molecules. Nevertheless, they subsequently escape the negative selection owing to specific co-stimulatory and cytokine signals which trigger the expression of the FoxP3 transcription factor. Then, developed natural Tregs migrate to the periphery (Hsieh et al., 2012).

In reverse, induced Tregs are generated in secondary lymphoid organs from $FoxP3^-CD4^+$ T cells under various conditions. In the periphery, Tregs differentiate under anti-inflammatory conditions. However, they can also be developed in the inflammatory environment when they differentiate quasi-simultaneously with effector T cells but at a lower rate (Bilate and Lafaille, 2012). For this reason, induced Tregs play a role in the suppression of the collateral damage during the eradication of the pathogens. They further regulate intestinal

immunity and microbes. It seems that induced Tregs also suppress allergic-type inflammation at mucosal surfaces (Dhamne et al., 2013).

Suppressive mechanisms of Tregs

Tregs exert their immunoregulatory functions via a considerable variety of mechanisms. The fundamental categories of these mechanisms include the production of soluble factors and the expression of inhibitory receptors on their surface. It seems that natural thymus-derived Tregs participate in immunoregulation more via cell-to-cell contact-dependent manner while induced Tregs modulate immune reactions in a contact-independent way (Jonuleit and Schmitt, 2003). Furthermore, Tregs display their immunosuppressive effects on a diverse range of immune cells, including different subsets of effector T cells, B cells, DCs, macrophages, natural killer (NK) cells, or mast cells (Shevach, 2009).

Tregs regulate immune reactions through three primary ways: the expression of inhibitory receptors, the production of soluble factors, and competition for growth factors (Bayati et al., 2021). These ways are schematically illustrated in Figure 1.

Primary inhibitory receptors which mediate Treg suppressive functions are cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), FasL, and lymphocyte activation gene 3 (LAG-3). CTLA-4 is a structural homolog of CD28 which both are ligands of CD80 and CD86 co-stimulatory molecules. CTLA-4 binds to these co-stimulatory molecules with higher affinity and avidity than CD28 molecule, which causes that CTLA-4^+ Tregs consume co-stimulatory signal from APCs that impairs effector T cell co-stimulation resulting in immunosuppression (Rowshanravan et al., 2018).

FasL represents a ligand of the Fas receptor, which triggers the signaling pathway leading to cell death via apoptosis (Volpe et al., 2016). FasL-mediated cell death plays a significant role in the downregulation of autoimmune responses via eliminating APCs or directly eliminating effector T cells (Gorbachev and Fairchild, 2010; Liu et al., 2017).

LAG-3 is a homolog of CD4 molecule binding MHCII molecules on APCs, thereby potentiating the suppressive function of Tregs (Huang et al., 2004). Depletion of LAG-3^+ Tregs leads to the development of experimental autoimmune encephalitis (EAE) in mice. It implies that the expression of LAG-3 on Tregs mediates the protection of the central nervous system from autoimmunity (Thaker et al., 2018).

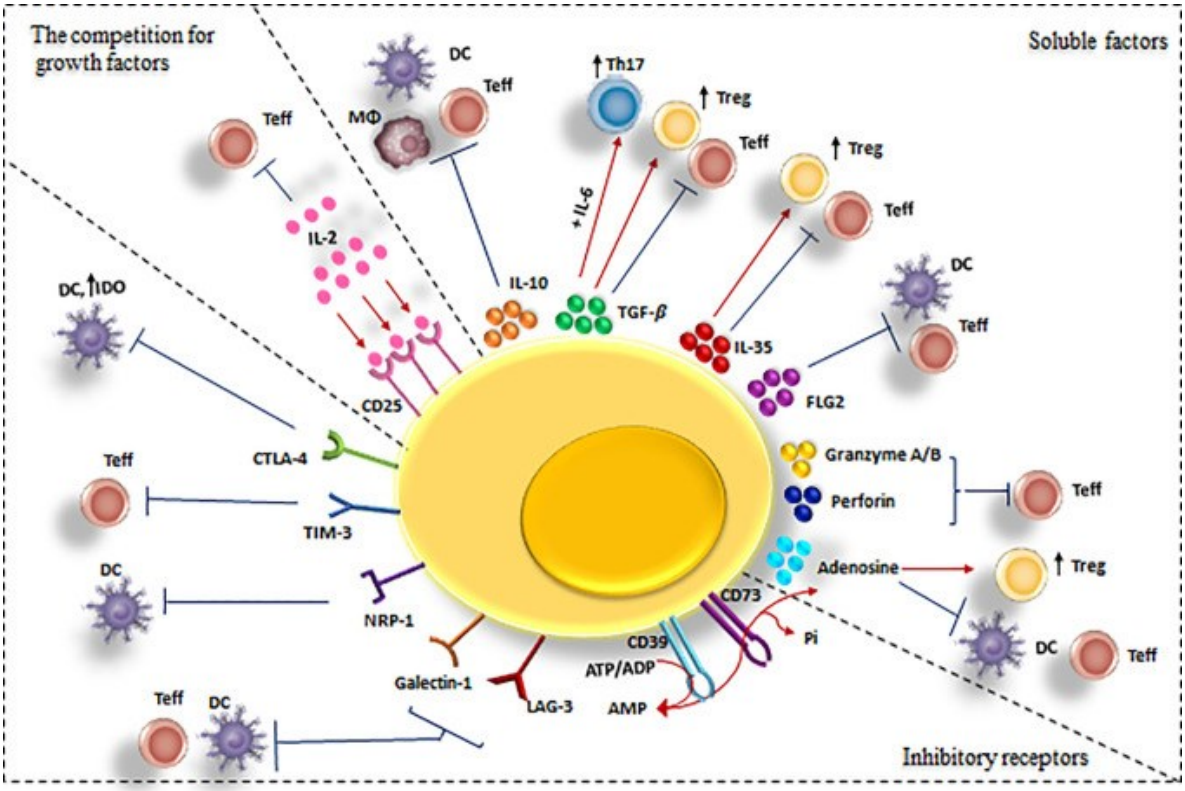

Figure 1: Suppressive mechanisms of Tregs. *Tregs have shown a variety of molecular mechanisms which participate in Treg-mediated suppression. There are three primary subcategories of Treg action:* ***1)*** *Production of soluble factors with anti-inflammatory and immunosuppressive effects including IL-10, TGF-β, IL-35, FLG2 (fibrinogen-like protein 2), granzymes, perforin, and adenosine;* ***2)*** *Expression of inhibitory receptors on Treg surface involving CTLA-4, TIM-3 (T cell immunoglobulin, and mucin domain-containing protein 3), NRP-1 (neuropilin-1), galectin-1, and LAG-3;* ***3)*** *Competition for IL-2, the pivotal growth factor of T cells, via expression of high-affinity CD25 molecule – an α chain of a receptor for IL-2 (adapted from Bayati et al., 2021).*

Among fundamental soluble factors by which Tregs modulate immune reactions belong to IL-10, TGF-β, IL-35, granzyme A/B, and adenosine. IL-10 is a cytokine with powerful anti-inflammatory and immunomodulatory effects. It binds to the heterodimeric IL-10 receptor, triggering signaling cascade of the signal transducer and activator of transcription 3 (Saraiva et al., 2020), resulting in transcription inhibition of pro-inflammatory nuclear factor κ B, which can downregulate the expression of MHCII molecules or co-stimulatory CD80 and CD86 molecules on the surface of APCs (Dorrington and Fraser, 2019; Schulke, 2018).

TGF-β is a pleiotropic cytokine that modulates multiple aspects of cellular functions, such as proliferation, differentiation, migration, or survival. The binding of TGF-β to the heterodimeric TGF-β receptor initiates signaling pathways resulting in the translocation of Smad transcription factor into the nucleus triggering the transcription machinery of targeted genes (Clarke and Liu, 2008). TGF-β blocks the development and differentiation of both T helper (Th) 1 and Th2 cells through several molecular mechanisms. In addition, it has a suppressive effect on the effector functions of T cells (Chen et al., 2003; Gorelik et al., 2000, 2002; Lin et al., 2005). Moreover, TGF-β supports the survival of natural Tregs and plays a role in the development of induced Tregs in the periphery (Li and Flavell, 2008).

IL-35 is a heterodimeric cytokine identified as a member of the IL-12 family with anti-inflammatory biological activity. IL-35 takes part in the expansion of Tregs, the inhibition of effector T cell proliferation, and prevention of Th17 polarization (Castellani et al., 2010). Nevertheless, it has been reported that simultaneously with IL-10, IL-35 can limit effective anti-tumor immune reactions (Sawant et al., 2019).

Granzymes A/B in cooperation with perforins drive the cytotoxicity and death of targeted cells. Oligomerization of perforin molecules generates perforin channels through which granzymes enter the cell and trigger a signaling pathway initializing apoptosis of the targeted cell (Arce-Sillas et al., 2016). This effect of Tregs is involved in attenuating lung inflammation during acute viral infection or tumorigenic growth (Choi et al., 2013; Loebbermann et al., 2012).

Extracellular adenosine triphosphate (ATP) acts as a pro-inflammatory signal which can be shifted toward an anti-inflammatory signal through cleavage of ATP to adenosine molecules. CD39 and CD73 molecules expressed on the Treg surface gradually generate adenosine molecules from extracellular ATP (Beavis et al., 2012; Vitiello et al., 2012).

Another elementary way of Treg suppressive function is the consumption of growth factors. It involves mainly competition for IL-2 between effector T cells and Tregs. IL-2 is essential for the activation and proliferation of effector T cells (Gasteiger and Kastenmuller, 2012). The expression of the α chain of IL-2 receptor (CD25 molecule) is constitutively upregulated on Tregs compared to effector T cells by which Tregs achieve more efficient binding and consumption of IL-2 (Pandiyan et al., 2007).

Treg role in autoimmunity, transplantation, and cancer

Tregs play a pivotal role in the maintenance of homeostasis and self-tolerance. More than 30 years from the discovery of Tregs, the involvement of dysregulation of Tregs has been

described in a wide variety of autoimmune diseases or allergies caused by the breakdown of tolerance (Dominguez-Villar and Hafler, 2018). It has been demonstrated that Tregs with impaired suppressive capacity occur in patients suffering from myasthenia gravis, MS, RA, or polyglandular syndrome compared to healthy individuals (Dejaco et al., 2006). In addition, decreased frequency of Tregs was described in patients with, for instance, psoriasis or T1D (Hull et al., 2017; Mattozzi et al., 2013). *Ex vivo* induction and expansion of Tregs and their adoptive transfer as a treatment of autoimmune disorders are studied in several clinical trials focused primarily on the treatment of T1D (https//:clinicaltrials.gov/).

The ability of Tregs to regulate immune reactions and attenuate inflammation displays their importance as critical players in the transplantation field. Already in 1990, Hall et al. showed the direct effect of $CD4^+CD25^+$ T cells on prolonging cardiac allograft survival. Generally, alloantigen presentation activates effector alloreactive T cells, which can cause graft damage and dysfunction. Tregs can limit GvHD by different immunosuppressive functions, also prevent graft rejection and facilitate transplantation tolerance (Walsh et al., 2004). The potential of Tregs to suppress alloreactions constitutes a promising therapeutic strategy that targets against graft reactions without a broad spectrum of adverse effects of immunosuppression (Atif et al., 2020). It is suggested that it may be achieved by *ex vivo* induction and expansion of Tregs and their subsequent application into patients (Romano et al., 2019). First-in-man studies using *ex vivo* prepared Tregs were already performed in solid organ transplantations. Several ongoing clinical trials study the effects of adoptive Treg transfer within liver or kidney transplantations (https//:clinicaltrials.gov/).

The immunosuppressive capacity of Tregs includes beneficial effects for the treatment of diseases or conditions when overreaction of the immune system impairs the homeostasis and healthy body physiology. On the other hand, potent immunoregulation might lead to the neglection of tumorigenic growth and cancer formation. Tumors build their own complex and dynamic microenvironment that forms suitable conditions for tumor growth and invasion (Baghban et al., 2020). This environment also influences migration and effector functions of immune cells, for instance, by the production of different growth and chemoattractant factors or vesicles (Lippitz, 2013). These conditions might cause migration and infiltration of Tregs into the tumor environment, which leads to suppression or prevention of tumor eradication. The involvement of Treg action in cancer immunity was already described in 1999. The administration of anti-CD25 monoclonal antibodies caused the regression of growing tumors in mice (Onizuka et al., 1999). Limitations of Treg effects, for example, Treg depletion, inhibition of their functions, or disruption of their migration into the tumor microenvironment,

represent a selective target for cancer therapy, resulting in boosting anti-tumor responses without substantial adverse effects (Plitas and Rudensky, 2020).

1.2.2.2. Regulatory B cells

Bregs represent a heterogeneous population of B cells with immunoregulatory effects. Almost 20 years before, it was described that B cells could also modulate immune responses in an antibody-independent manner (Mizoguchi et al., 2002). Like Tregs, Bregs exert many different types of immunomodulatory functions and play a role in maintaining immunological tolerance and homeostasis (Abebe et al., 2021).

The increasing number of studies provide evidence that Bregs contribute to ameliorating autoimmune disorders to reduce inflammation and support tolerogenic mechanisms leading to graft acceptance in transplantation. Nevertheless, Breg suppressive functions can impair anti-tumor immune responses, and as a result, they may enhance the promotion of cancer growth, tumor invasion, and metastasis (Abebe et al., 2021).

Origin, phenotype, and markers

Although many groups have studied the origin and phenotype of Bregs, there remains still controversy and unclarity about Breg specific precursor and specific marker (Ran et al., 2020). The fundamental characteristic markers of Bregs are their immunoregulatory functions and ability to suppress inflammatory reactions (Rosser and Mauri, 2015). Nevertheless, the CD9 molecule was described as a shared Breg marker, but the only particular fraction of $CD9^+$ B cells has the capacity to produce IL-10 (Mohd Jaya et al., 2021; Sun et al., 2015). Further, in several studies, the FoxP3 transcription factor was also suggested as a possible Breg marker. However, its expression and role in B cells remain controversial (Noh et al., 2012; Park et al., 2016; Vadasz et al., 2015; Vadasz and Toubi, 2017). Additionally, it has also been described that hypoxia-inducible factor-1α (HIF-1α) can be effective for IL-10-producing Bregs recognition (Meng et al., 2018). Despite that, there is still none of a generally accepted Breg marker. Besides, Bregs might develop from various B cell subpopulations; thus, they are commonly characterized based on overlapping markers with conventional B cell subsets (Cherukuri et al., 2021).

Conventional B cells can be divided into B1 and B2 cells. B1 cells can be further distinguished according to the expression of CD5 molecule to B1a cells ($CD5^+$) and B1b cells ($CD5^-$). B2 cells can be classified as follicular (FO) and marginal zone (MZ) B cells that differ in the localization and the expression of surface markers. FO B cells are mainly found in

lymphoid follicles of secondary lymphoid tissues or in circulation, whereas MZ B cells are usually located in the MZ of the spleen. When bone marrow-derived immature B cells undergo the maturation process, they go through different phases of maturation. These B cells are called transitional B cells (Sagaert and De Wolf-Peeters, 2003; Yanaba et al., 2009).

Numerous studies suggested that Bregs do not represent a population of B cells that develop from one common progenitor. Due to the enormous heterogeneity of Breg phenotypes, which overlap with classical B cell populations, it seems that Bregs can develop from different B cell subtypes in response to local stimuli and microenvironment (Amu et al., 2010; Blair et al., 2010; Chao et al., 2016; Ding et al., 2011; Iwata et al., 2011; Ronet et al., 2010). Possible development pathways, including surface markers of Breg subsets, are illustrated in Figure 2.

The local milieu is pivotal for Breg differentiation. Many different B cell populations can develop suppressive Breg phenotype following endogenous or exogenous stimuli. Bregs often expand under inflammatory conditions, which involve signalization through toll-like receptors (TLR), BCR, CD40 molecules, or CD80 and CD86 co-stimulatory molecules (Fillatreau et al., 2002; Lampropoulou et al., 2008; Menon et al., 2021; Yanaba et al., 2008). Bregs can also develop in the microenvironment of inflammatory cytokines (Mohd Jaya et al., 2019). For instance, the cooperation of *Schistosoma mansoni* eggs, IL-4 and IL-10 was important for the effective induction of FasL expression in B cells (Lundy and Boros, 2002). Further, it was shown that IL-1β and IL-6 can play a role in Breg differentiation. IL-6 receptor and IL-1β receptor double knockout mice exerted the decreased number of IL-10-producing B cells and developed a severe RA form compared to control mice (Rosser et al., 2014). In addition, Bregs also expanded after the stimulation by pro-inflammatory IL-21 cytokine, and IL-21 also enhanced the IL-10 secretion by B cells (Yoshizaki et al., 2012). On the other hand, the costimulation of B cells by lipopolysaccharide (LPS) and IL-21 impaired their IL-10 production. Nevertheless, the costimulation by LPS and interferon (IFN)-γ increased IL-10 production by B cells (Holan et al., 2014). It was also described that the administration of IL-33 into the peritoneal cavity of mice increased the proportion of IL-10-producing cells. Subsequently, the adoptive transfer of these cells into IL-$10^{-/-}$ mice blocked the development of mucosal inflammatory responses in the gut (Sattler et al., 2014). IFN-α produced by plasmacytoid DCs could also induce the Breg development, and consequently, IL-10 secretion by B cells controlled the activity of plasmacytoid DCs. In patients suffering from systemic lupus erythematosus (SLE), this crosstalk between B cells and plasmacytoid DCs was impaired, contributing to SLE pathology (Menon et al., 2016). IL-35 was capable of inducing the Breg

development as well. As a result, Bregs attenuated the severity of autoimmune uveitis (Dambuza et al., 2017; Wang et al., 2014).

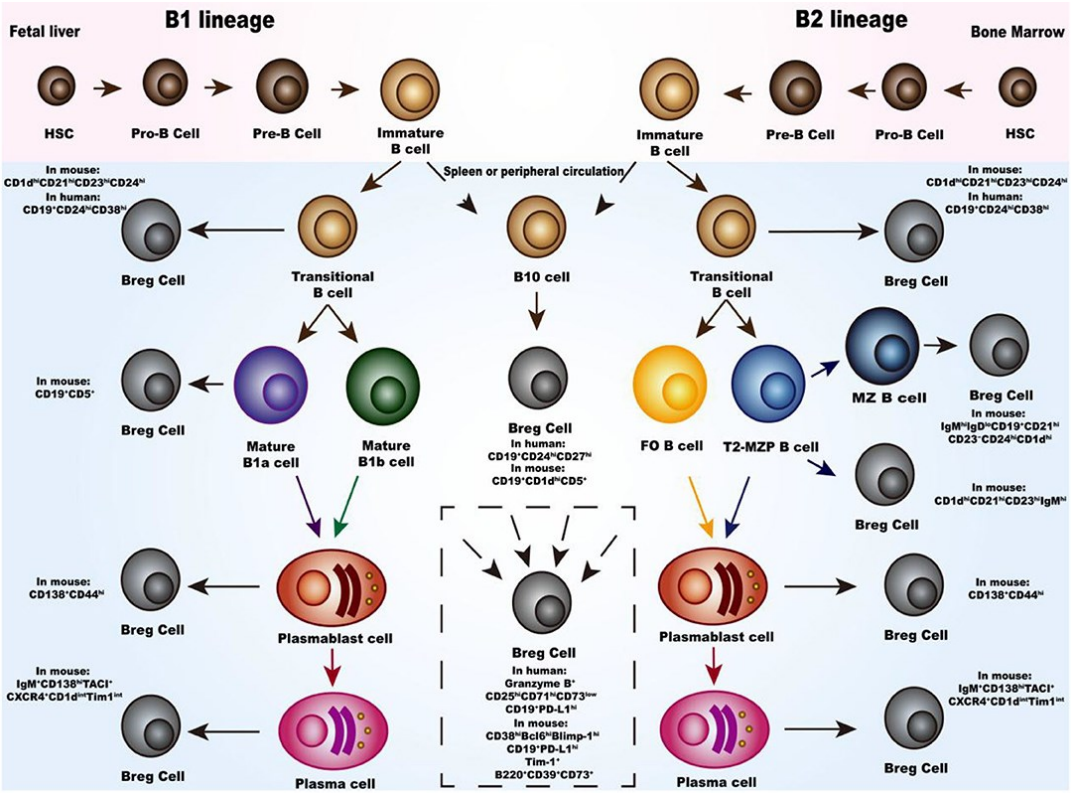

Figure 2: Development and differentiation of Bregs. *B cells develop into immature B cells from hematopoietic stem cells in the bone marrow. Further, they differentiate through a stage of the transitional B cell to mature B cells. Immature B1 cells mature into either B1a or B1b subpopulations which can consequently differentiate into plasma cells. In contrast, immature B2 cells develop into either FO (follicular) or MZ (marginal zone) B cells (with the intermediate step of T2-MZP (transitional-2-marginal zone precursors) B cells), which can eventually undergo terminal differentiation into plasma cells. Many references proved that, based on overlapping phenotype, IL-10-producing Bregs can differentiate from transitional B cells, MZ B cells, T2-MZP B cells, B1 cells, plasmablast cells, or plasma cells. Moreover, Breg phenotypes cannot be described according to standard B cell classification because there is a lack of studies (adapted from Ran et al., 2020).*

Taken together, Bregs can differentiate from distinct populations of classical B cells in dependence on the local milieu. Bregs share characteristic markers with the B cell subset from which they arise. The development of Bregs is triggered by inflammatory stimuli, which predict

the role of Bregs as a negative feedback loop in immune responses, avoiding self-harming immune reactions.

Suppressive mechanisms of Bregs

Bregs exert their suppressive effects via a vast range of mechanisms. These mechanisms involve the production of anti-inflammatory cytokines as well as cell-to-cell contact-dependent actions. Bregs manifest the ability to produce IL-10, TGF-β, IL-35 and, in addition, to express FasL, PD-L1/2, TRAIL, a ligand of glucocorticoid-induced tumor necrosis factor receptor (GITR), and CD39 and CD73 molecules. Nevertheless, the hallmark mechanism of Bregs is the production of the anti-inflammatory cytokine IL-10 (Ray et al., 2012; Sarvaria et al., 2017; Wasik et al., 2018). Figure 3 schematically displays the main immunosuppressive effects of Bregs.

The first described and critical mechanism of Breg action is the production of IL-10 (Mizoguchi et al., 2002). IL-10 is a central anti-inflammatory cytokine that can suppress the differentiation, proliferation, activation, and terminal functions of many different immune cells such as T cells, macrophages, or NK cells (Couper et al., 2008). The beneficial effect of IL-10-producing Bregs was described in various autoimmune diseases, such as EAE, SLE, RA, or contact dermatitis (Blair et al., 2009; Fillatreau et al., 2002; Flores-Borja et al., 2013; Yanaba et al., 2008).

An additional soluble factor by which Bregs influence immune responses is TGF-β which participates in immunomodulation and immunosuppression. Besides, it affects the proliferation, differentiation, activation, or survival of effector immune cells (Clarke and Liu, 2008). TGF-β-producing Bregs ameliorate manifestations of T1D, experimental asthma, or autoimmune neuroinflammation in mice (Bjarnadottir et al., 2016; Natarajan et al., 2012; Tian et al., 2001). Furthermore, these Bregs participate in the induction of transplant tolerance (Lee et al., 2014; Peng et al., 2018).

Bregs are also capable of the production of IL-35, which contributes to immunoregulation. IL-35 exhibits immunosuppressive function via the inhibition of proliferation, activation, and cytotoxicity of pathological effector T cells and the induction of Treg differentiation (Huang et al., 2017; Kourko et al., 2019). IL-35-producing Bregs can play a role in controlling autoimmune, degenerative disorders of the central nervous system. It was shown that expansion of IL-35-producing Bregs ameliorates experimental autoimmune uveitis, EAE, or MS (Choi and Egwuagu, 2021; Shen et al., 2014).

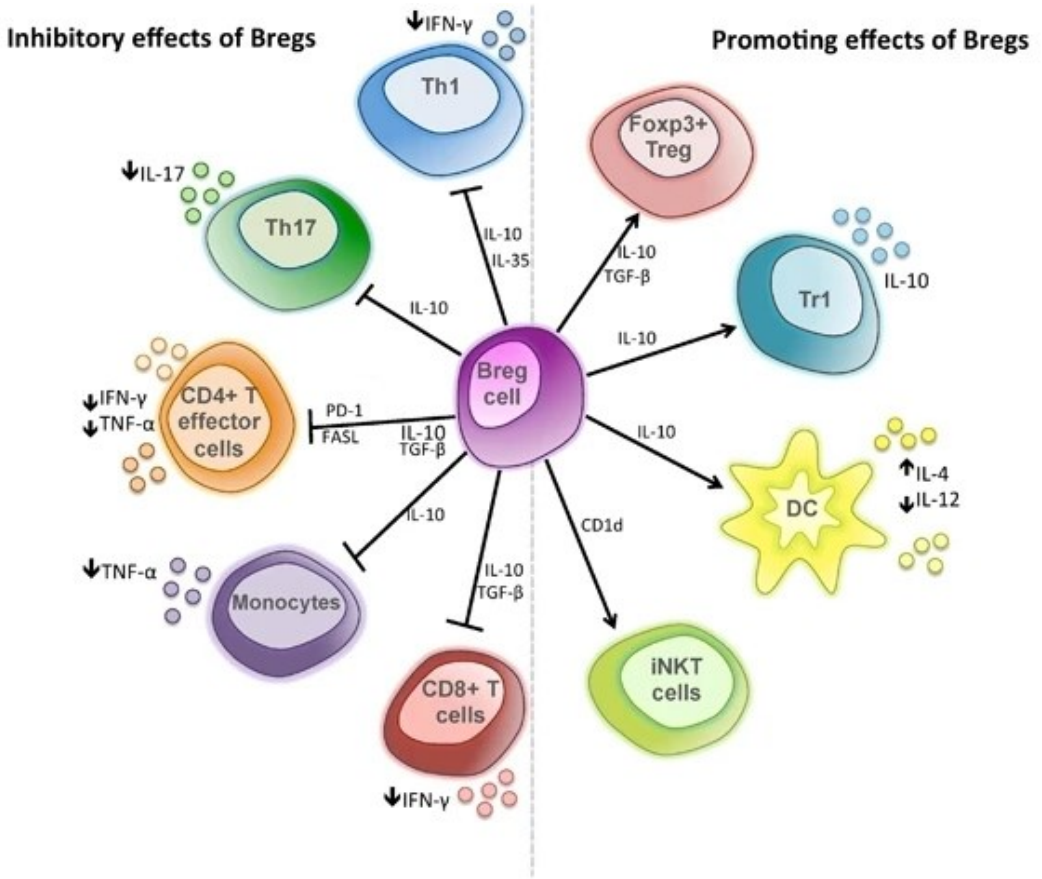

Figure 3: Suppressive mechanisms of Bregs. *Bregs affect different immune cells and exhibit inhibitory or promoting effects depending on a terminal effector cell. Bregs perform their immunoregulatory functions via a variety of mechanisms. These mechanisms are mediated by releasing soluble molecules, such as IL-10, IL-35, or TGF-β. Bregs can also influence immune cells via direct cell-to-cell contact. On the surface, Bregs express, for instance, FasL or PD-L1 molecules (adapted from Sarvaria et al., 2017).*

Bregs can also mediate their immunosuppressive effects independently of cytokine secretion by direct cell-to-cell contact. Like Tregs, Bregs can express FasL molecules that trigger signaling pathways leading to cell death (Volpe et al., 2016). Effects of $FasL^+$ Bregs are mediated via blocking of the proliferation of $CD4^+$ T cells and supporting apoptosis of effector T cells by which Bregs ameliorate contact hypersensitivity, T1D, or collagen-induced arthritis (CIA) (Lundy and Fox, 2009; Tian et al., 2001; Wang et al., 2017a). Bregs can also express additional molecules which initiate the cell death, PD-L1/2 molecules. The binding of PD-L1/2 molecules mediates the inhibition of T cell expansion and termination of their effector function, such as cytokine production or cytotoxicity (Khan et al., 2015). Further, $PD\text{-}L1^+$ Bregs support the development of Tregs (Said et al., 2018). Another death-ligand expressed on Bregs is TRAIL. Bregs promote the expression of TRAIL after stimulation with

cytosine-phosphate-guanine motive oligonucleotides or IFN-α. These Bregs then were capable of induction of apoptosis in melanoma cells (Kemp et al., 2004).

Furthermore, Bregs can express the ligand of GITR, which engages the activation and survival of immune cells and their signaling (Wang et al., 2021). Bregs can induce Treg differentiation and proliferation through GITRL/GITR signaling (Ray et al., 2012).

Finally, the generation of adenosine is another immunosuppressive mechanism of Bregs. Due to Breg's ability to express CD39 and CD73 molecules, they can switch pro-inflammatory signals to anti-inflammatory by cleaving ATP to adenosine (Allard et al., 2017). The administration of $CD73^+$ Bregs ameliorates the manifestation of colitis in the mouse model (Kaku et al., 2014).

Altogether, Bregs exhibit their immunosuppressive effect via a wide variety of mechanisms, including the production of soluble factors and expression of surface markers down-regulating the expansion and functions of pro-inflammatory immune cells.

Breg role in autoimmunity, transplantation, and cancer

Bregs play an essential role in immunoregulation. It has been shown that they can support the mechanisms of transplantation tolerance and suppress harmful redundant immune reactions in autoimmune diseases or antiviral defense. On the other hand, effective suppression of immune reactions mediated by Bregs can lead to impairment of immune surveillance and tumor generation (Catalan et al., 2021).

Accumulating evidence suggests that Bregs have a pivotal effect on the regulation of autoimmune diseases described in various models. The protective functions of Bregs against autoimmune reactions are exhibited via different mechanisms involving cell-to-cell contact-dependent or independent effects. Decreased severity of chronic intestinal inflammation is associated with the production of IL-10 by Bregs (Mizoguchi et al., 2002). IL-10-producing Bregs are also able to ameliorate manifestation of EAE, SLE, or MS (Fillatreau et al., 2002; Knippenberg et al., 2011; Wang et al., 2017b). Further, Bregs expressing PD-L1 molecules influence the activity of RA or EAE (Carter et al., 2007; Cui et al., 2015). Although B cells can impair pancreatic β cells by the production of autoreactive antibodies, Bregs can mediate protective effects via FasL expression and TGF-β production against tissue damage (Tian et al., 2001).

Additionally, studies showing Bregs differentiation and induction during bacterial, viral, or parasitic infections support Breg's role as a negative feedback loop in immune reactions. For example, mice with B-cell-specific deletion of the gene for IL-35 had improved resistance to

Salmonella typhimurium infection (Shen et al., 2014). Similarly, patients with chronic hepatitis B infection had an increased number of Bregs in peripheral blood, which potentiated the activity of Tregs and attenuated effector T cell functions (Liu et al., 2016). Bregs performed beneficial effects during severe malaria because they prevent overreactions of cytotoxic T cells (Liu et al., 2013).

Breg ability to create a tolerogenic environment might be helpful in the prevention of graft rejection and GvHD. For instance, patients with chronic rejection after kidney transplantation exhibited a reduced proportion of $CD19^+CD24^+CD38^+$ Bregs in peripheral blood (Silva et al., 2012). Additionally, it was also reported that patients suffering from chronic antibody-mediated rejection had impaired immunoregulatory functions of Bregs. These cells could not effectively suppress the response of $CD4^+$ effector T cells after kidney transplantation. Interestingly, Bregs achieved an immunosuppressive effect on T cells via granzyme B-dependent pathway (Chesneau et al., 2015).

In cancer circumstances, immunosuppressive properties of Bregs perform undesirable and harmful reactions resulting in the reduction of anti-tumor response by pro-inflammatory effector immune cells. Firstly, it was described that endogenous Bregs limited the eradication of lymphoma cells in anti-CD20 immunotherapy (Horikawa et al., 2011). Increased proportion of circulated Bregs is also linked with the promotion of tumor development, growth, invasiveness, and recurrence of hepatocellular carcinoma directly via the CD40/CD40L signaling pathway (Shao et al., 2014). Moreover, TGF-β-producing Bregs can evoke lung metastasis in breast cancer by promoting Treg differentiation and proliferation (Olkhanud et al., 2011).

Bregs play a central role in health and disease orchestra. Their powerful immunoregulatory functions represent possible therapeutic targets that can pave the way for new and improved therapeutic strategies.

1.3. Mesenchymal stem/stromal cells

Mesenchymal stem/stromal cells (MSCs) represent a heterogeneous population of multipotent stromal cells with a self-renewal capacity and multilineage differentiation ability (Fernandez-Francos et al., 2021). Further, MSCs exert regenerative and immunomodulatory potential. The regenerative potential of MSCs is based predominantly on the production of a broad spectrum of growth and nutrition factors and on their ability to differentiate or transdifferentiate into other cell types. Beyond the production of molecules supporting

regeneration, MSC can also express a variety of immunomodulatory molecules that allow MSCs to perform immunoregulatory properties. MSCs regulate the activity and functions of both innate and adaptive immune systems, including the reactivity of complement, DCs, monocytes, and macrophages, granulocytes, or NK cells. Above that, they also influence B and T cell differentiation and terminal functions (Song et al., 2020; Weiss and Dahlke, 2019). The main effects of MSCs on immune cells are summarized in Figure 4. Nevertheless, it is important to mention that the culture conditions of MSCs can influence their properties, and their immunoregulatory capacity might be decreased; otherwise, immunogenicity might rise (Liu et al., 2012).

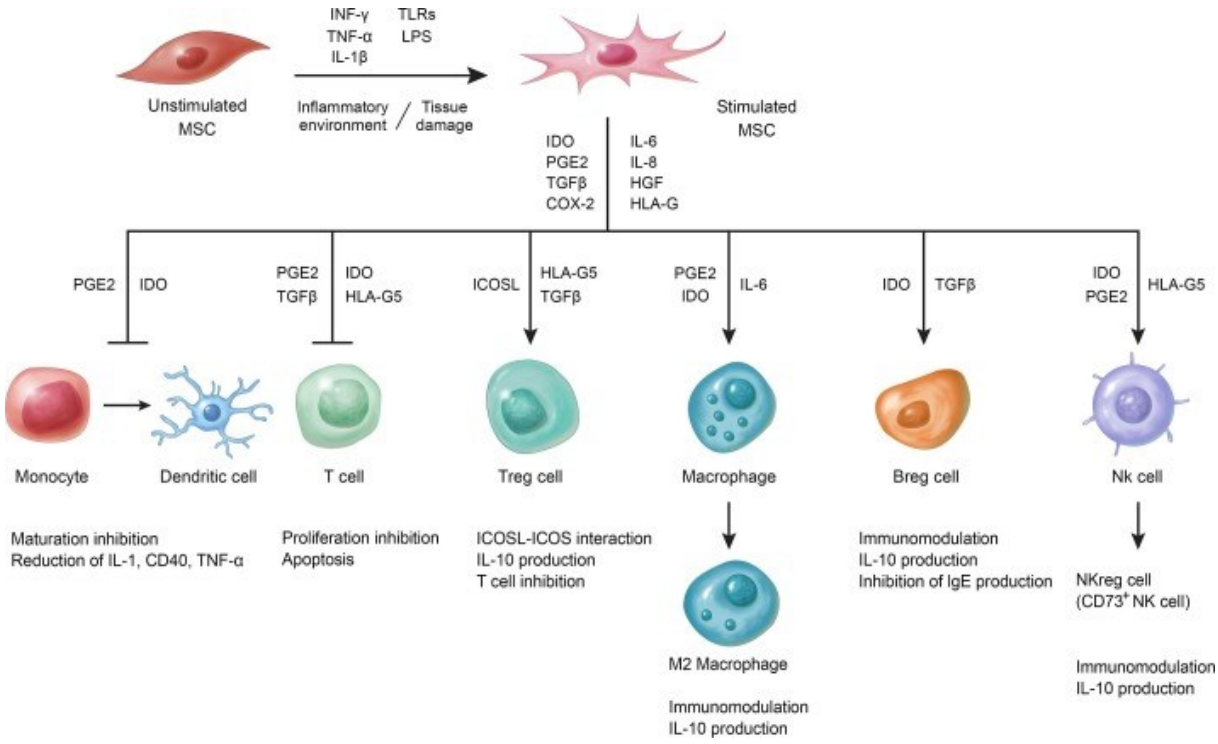

Figure 4: Immunoregulatory effects of stimulated MSCs. *MSCs exhibit their immunomodulatory properties by expressing various suppressive factors that can influence the cells of innate and adaptive immune systems. MSCs inhibit differentiation, proliferation, and effector functions of pro-inflammatory immune cells. On the other hand, MSCs promote the shift of immune cells into their regulatory subtypes (adapted from Lee and Song, 2018).*

MSC origin and markers

MSCs were first described in 1966 as bone marrow cells with osteogenic potential by Friedenstein et al. Later, it has been shown that MSCs can be found and isolated from various tissues of the adult organism, such as bone marrow, blood, adipose tissue, muscles, dermis, synovial membrane, etc. (Marquez-Curtis et al., 2015). Nevertheless, there has not been found a unique phenotypical marker for MSCs so far. Therefore, phenotypic MSC characterization is based on the expression of several surface markers and the lack of hematopoietic markers (Lin et al., 2013).

As a result of the increasing number of studies of MSCs isolated from different sources by distinct approaches, the Mesenchymal and tissue stem cell committee of the International Society for cellular therapy proposed minimal criteria for MSC defining (Dominici et al., 2006). Firstly, MSCs must be plastic-adherent. Secondly, MSCs must be able to differentiate into osteoblasts, adipocytes, and chondroblasts under specific culture conditions. Moreover, finally, human MSCs must express CD105, CD73, and CD90 surface molecules and be negative for CD45, CD34, CD14, or CD11b, CD79a, or CD19, and human leukocyte antigen (HLA)-DR molecules. The cells must meet all criteria to be able to describe them as MSCs.

In 2019 this international committee further clarified the MSC nomenclature (Viswanathan et al., 2019). The interchangeable usage of the acronym MSCs as mesenchymal stem cells and mesenchymal stromal cells led to patient confusion due to the disused term stem cells. Patients assume that they gain the direct therapeutic benefits of MSC differentiation into physiological tissue. Nevertheless, many studies disuse the acronym MSCs in meaning "mesenchymal stem cells," although these cells should be called "mesenchymal stromal cells" according to the original committee statement. Furthermore, the committee highlighted the recommendation to declare cell source, indicating MSCs as mesenchymal stromal cells, unless *in vitro* and *in vivo* data provide clear evidence for stemness. The committee also recommended accomplishing a variety of functional assays to demonstrate MSCs characteristics accurately.

1.4. Interactions of MSCs and immunoregulatory cells

Beyond the differentiation potential of MSCs, they exert a broad scale of different mechanisms by which they can module the reactivity of immune cells of the immune system. MSCs produce various anti-inflammatory and immunoregulatory soluble factors and express immunomodulatory molecules on their surface to control immune reaction via cell-to-cell contact-dependent manner (Weiss and Dahlke, 2019).

The production of immunoregulatory molecules by MSCs involves the secretion of TGF-β (Liu et al., 2019), IL-6 (Mi and Gong, 2017), nitric oxide produced by inducible nitric oxide synthase (iNOS) (Maria et al., 2018), prostaglandin E2 (PGE_2) released by cyclooxygenase-2 (COX-2) (Kota et al., 2017), HLA-G5 in humans (Selmani et al., 2008), tumor necrosis factor-α stimulated gene 6 (Li et al., 2018), hepatocyte growth factor (Kennelly et al., 2016) and intracellular enzyme IDO which catabolizes tryptophan (Lim et al., 2021). Moreover, immunomodulation by MSC-derived exosomes has also been studied. These vesicles can interact with targeted cells through cell receptors, and they are also loaded by growth factors, cytokines, nucleic acids, and additional immunomodulatory factors shifting the targeted cell metabolism (Nikfarjam et al., 2020).

MSC-derived particles mediating the suppression of immune reactions via cell-to-cell contact include PD-L1 and FasL molecules which can participate in immunoregulation by eliminating overreactive immune cells. Thus, MSCs can decrease the number of pro-inflammatory Th17 cells via PD-L1 molecules or promote the apoptosis of activated immune cells and inhibit harmful immune reactions through FasL (Akiyama et al., 2012; Davies et al., 2017).

Modulation of MSC properties

MSCs can express the immunomodulatory molecules either constitutively or as a result of defined stimulation (Najar et al., 2019). It clearly shows that MSC properties are extensively influenced by their local milieu. This has a crucial effect on the therapeutic potential of MSCs because priming of MSCs by cytokines, TLR ligands, or drug preincubation can considerably affect MSC properties before their administration. It was described that it is possible to enhanced MSC production of immunoregulatory molecules by the stimulation using IFN-γ, tumor necrosis factor-α, IL-1β, or their combination (Kim et al., 2018; Redondo-Castro et al., 2017; Yan et al., 2018). On the other hand, the stimulation of MSCs through TLR can alter their properties according to the specific type of TLR. TLR4 engagement provided MSC polarization toward pro-inflammatory signature. On the contrary, the priming of MSCs by ligands of TLR2 and 3 promoted the MSC anti-inflammatory characteristics (Waterman et al., 2010).

Similarly, the properties of MSCs can also be affected by immunomodulators, such as immunosuppressive drugs, administrated simultaneously during MSC therapy because combined administration is essential for treatment protocol success (Peng et al., 2013). Nevertheless, the targeted molecules of immunosuppressive drugs are also expressed by MSCs, affecting MSC properties. It has been described that glucocorticoids can enhance the IDO

expression in MSCs (Ankrum et al., 2014). Further, rapamycin can also augment the expression of IDO, IL-10, and TGF-β and concurrently decrease IL-6 and IL-1β expression. Moreover, rapamycin-primed MSCs ameliorated manifestation of GvHD in a mouse model via suppression of Th1 and Th17 reactions and support of Th2 and Treg development (Kim et al., 2015).

Additionally, it was demonstrated that MSCs have the ability to attach the immunosuppressants on their surface, which can improve the therapy of immunomodulatory drugs due to the capability of MSCs to migrate selectively to the inflammatory environment (Girdlestone et al., 2015). Recently published studies revealed that combined therapy of MSCs and immunosuppressive drugs can represent favorable interaction for the long-term control of the immune system (Javorkova et al., 2018; Kaundal et al., 2018). Nevertheless, immunomodulatory drugs can slightly impair cytokine secretion and migration of MSCs; some authors recommend the washout period between the administration of immunosuppressants and MSCs according to half-live of the drugs (Lightner et al., 2019).

MSCs play a pivotal role in immunomodulation and represent negative regulators of immune reactions because a pro-inflammatory environment can induce the production of anti-inflammatory molecules by MSCs. However, the local environment that leads to MSC priming can strongly alter MSC properties, which must be considered.

1.4.1. MSCs and Tregs

MSCs regulate proliferation, differentiation, cytokine production, and effector functions of T cells and their subpopulations - $CD4^+$, $CD8^+$ T cells, Th17 cells, and Tregs (Weiss and Dahlke, 2019). Naïve $CD4^+$ Th cells may differentiate into several lineages of Th cells involving Th1 cells expressing a T-box transcription factor (T-bet), Th2 cells expressing GATA binding protein 3 (GATA-3), Th17 cells expressing retinoic-acid-receptor-related orphan nuclear receptor γ (RORγt), or $FoxP3^+$ Tregs (Golubovskaya and Wu, 2016). This development is dependent on a particular cytokine milieu. For almost each T cell subpopulation, the key polarization cytokine or combination is known (Golubovskaya and Wu, 2016). MSCs can alter the cytokine milieu, thereby affecting the polarization of particular Th cell subsets and shifting immune reaction from pro-inflammatory to anti-inflammatory (Duffy et al., 2011).

Substantial levels of TGF-β are important for Treg development (Wang et al., 2020). TGF-β may cause suppression of expression of the IL-23 receptor, limiting the function of

transcription factor RORγt leading to the promotion of Treg differentiation. Otherwise, decreased concentration of TGF-β under IL-6, IL-21, and IL-23 conditions induces polarization toward Th17 cells (Bovenschen et al., 2011; Zhou et al., 2008). Due to the capability of MSCs to produce both TGF-β and IL-6, they can alter the TGF-β and IL-6 ratio in the microenvironment and shift the Th17 cell toward Treg differentiation (Svobodova et al., 2012). Moreover, MSCs can support Treg differentiation via other soluble factors, such as PGE_2 (Hsu et al., 2013), HLA-G5 (Selmani et al., 2008), IL-10 (Naserian et al., 2020), hepatocyte growth factor (Chen et al., 2020a), or via expression of IDO (Gazdic et al., 2018). Additionally, MSCs can modify Th17 and Treg polarization via cell-to-cell contact by expressing the CD54 molecule, which triggers signalization through CD11a/CD18 molecules on Th17 cells, leading to induction of regulatory phenotype in Th17 cells (Ghannam et al., 2010).

Further, MSCs can alter Treg differentiation via APCs. MSC effects may enhance the number of Mregs (Hyvarinen et al., 2018), Bregs (Liu et al., 2020), and tDCs (Boks et al., 2012; Lu et al., 2019), which can result in the induction of Treg development. MSCs can also induce T cell apoptosis directly via FasL; apoptotic vesicles subsequently trigger TGF-β production by macrophages, achieving Treg induction (Akiyama et al., 2012).

The ability of MSCs to attenuate pathological reactions by activation of Tregs has been demonstrated in several models of autoimmune diseases, such as EAE (Ahmadvand Koohsari et al., 2021), experimental colitis (Lim et al., 2021), SLE (Wang et al., 2017c), Crohn´s disease (Ciccocioppo et al., 2011), or RA (Ghoryani et al., 2019). The additional mechanism involved in immunomodulatory MSC capacity is the induction of Tregs. It has been confirmed in the skin (Khosravi et al., 2018), kidney (Erpicum et al., 2019), or liver (Shi et al., 2017) allogeneic transplantation.

1.4.2. MSCs and Bregs

Recent studies have shown that MSCs also modulate the activation and reactivity of Bregs. Accumulating evidence has demonstrated that MSC administration alters B cell physiology toward the induction of Bregs. MSCs can regulate B cells via soluble factors and cell-to-cell contact (Liu et al., 2020).

Compared with the MSC generation of Tregs, the specific effects of MSCs on the modulation of Breg induction are still unclear. It has been proposed that the production of PGE_2 molecule via the COX-2 pathway by MSCs can generate $CD23^+CD43^+$ Bregs, which attenuate the severity of colon inflammation resulting in limitation of gastrointestinal mucosal tissue

pathology in a mouse model of colitis (Chen et al., 2019). It has been recently shown that MAPKs/AKT-AP1 signaling, or aryl hydrocarbon receptor pathway, which triggered the COX-2/PGE_2 axis in MSCs, achieves the induction IL-10^+ Bregs (Chen et al., 2020b). Nevertheless, IFN-γ-primed MSCs can suppress the IL-10 production of Bregs via the COX-2 pathway (Hermankova et al., 2016). In addition, MSCs can express Epstein-Barr virus-induced 3 protein, one of the subunits of anti-inflammatory cytokine IL-35. This production shift differentiation of B cells toward IL-10-producing Bregs instead of activating effector antibody-producing B cells (Cho et al., 2017).

Further, IL-10-producing $CD1d^{high}CD5^{high}CD83^{low}$ Bregs were expanded by mesenchymal-like stromal cells in a model of mouse cardiac allograft. The donor-specific tolerance depended on stromal cell-derived factor-1 (SDF-1) (Lan et al., 2017). On the other hand, the axis of SDF-1α and its C-X-C motif chemokine receptor 7 (CXCR7) revealed a dual effect of MSCs on IL-10-producing Bregs. Low concentrations of SDF-1α promoted IL-10^+ Breg expansion by MSCs, whereas high concentrations suppressed MSC-mediated Breg induction. Moreover, this reverse effect could be buffered by overexpression of CXCR7 (Qin et al., 2015).

MSCs can also play a role in Breg activation by the expression of IDO. Human umbilical cord-derived MSCs can enhance the expansion of $CD5^+$IL-10^+ Bregs and boost their functions, resulting in the alleviation of EAE (Li et al., 2019). In addition, MSCs can use the IDO pathway to promote the proportion of $CD5^+$ Bregs by increased Breg proliferation and survival (Peng et al., 2015).

In addition to the production of soluble factors, MSCs can influence Breg development via cell-to-cell contact. Although cell-to-cell contact-dependent manner of MSCs and Breg activity was shown using trans-well tissue culture system, particular molecules are not clearly described (Chen et al., 2019). Nevertheless, it seems that molecule PD-L1 can be involved in the MSC direct mechanism affecting IL-10-producing Bregs (Wang et al., 2014).

Several studies have described that the regulation of Bregs by MSCs can play a crucial role in various immune disorders. For the first time, Guo et al. (2013) described that two doses of MSCs injected intravenously led to suppression of EAE severity. This effect was associated with the increased Breg proportion (Li et al., 2019). Expansion of Bregs induced after injection of MSCs was also described in the mouse model of SLE, where the development of Bregs resulted in amelioration of autoimmunity (Park et al., 2015). Further, the administration of MSCs followed by an increased proportion of Bregs ameliorated the pathological severity of CIA (Yan et al., 2017). Moreover, the promotion of Bregs by MSCs was also described in the

model of GvHD (Peng et al., 2015). Accumulating evidence refers to MSC immunomodulatory capacity can ameliorate the spectrum of autoimmune diseases and alloreactions via the support of Breg development.

2. Aims

The general aim of this thesis has been to study different approaches to the modulation of the immune response, focusing on the induction, expansion, and activation of immunoregulatory cells. Presented work also highlights the necessity of accurate phenotypic characterization of immune cells to avoid of false results. The aims can be divided into the following fields of interest:

- **To analyze the effects of combined action of MSCs and immunosuppressive drugs on the balance of T cell subpopulations.** Immunosuppressive drugs have been designed to decrease undesirable overreactions of leukocytes but simultaneously display a range of different adverse effects. We analyze the impact of MSCs in combination with immunosuppressive drugs on the proportion of Th1/Th2/Th17 and Treg cells and their cytokine production.

- **To characterize the effects of cytokine-primed MSCs on Breg development.** Priming of MSCs can influence their immunomodulatory properties, and primed-MSCs can further modify the induction and terminal functions of B cells. We studied the impact of IFN-γ- or IL-4-primed MSCs on the development of IL-10-producing Bregs. Additionally, we focused on the description of signaling pathways involved in the effects of cytokine-primed MSCs.

- **To study the effects of cytokines on the development and phenotype of Bregs.** Cytokine milieu represents a master regulator of the induction, activation, and functions of individual subpopulations of immunoregulatory cells. Thus, we analyzed the effects of different cytokines on Breg and Treg development and their phenotype. We aimed to describe the modulatory impact of the selected cytokines on the activation of the suppressive functions of Bregs and their transcription factors.

- **To highlight inaccuracy in the immunophenotypical analysis of Breg populations.** Flow cytometry analysis is a powerful tool for distinguishing individual cell populations, but false-positive or false-negative results can impair it. We evaluated the discrepancies in the expression of transcription factors of Bregs between different studies.

3. List of publications

3.1. Publications

The thesis was prepared based on these publications:

Bohacova P, Kossl J, Hajkova M, Hermankova B, Holan V, Javorkova E. Difference between mitogen-stimulated B and T cells in nonspecific binding of R-phycoerythrin-conjugated antibodies. J Immunol Methods. 2021;493:113013.

Bohacova P, Kossl J, Hajkova M, Hermankova B, Javorkova E, Zajicova A, Krulova M, Holan V. Interleukin-10 production by B cells is regulated by cytokines, but independently of GATA-3 or FoxP3 expression. Cell Immunol. 2020;347:103987.

Hajkova M, Hermankova B, Javorkova E, **Bohacova P**, Zajicova A, Holan V, Krulova M. Mesenchymal stem cells attenuate the adverse effects of immunosuppressive drugs on distinct T cell subpopulations. Stem Cell Rev Rep. 2017a;13:104-115.

Holan V, Hermankova B, **Bohacova P**, Kossl J, Chudickova M, Hajkova M, Krulova M, Zajicova A, Javorkova E. Distinct immunoregulatory mechanisms in mesenchymal stem cells: Role of the cytokine environment. Stem Cell Rev Rep. 2016;12:654-663.

3.2. Other impacted publications

Kossl J, **Bohacova P**, Hermankova B, Javorkova E, Zajicova A, Holan V. Antiapoptotic properties of mesenchymal stem cells in a mouse model of corneal inflammation. Stem Cells Dev. 2021;30:418-427.

Holan V, Echalar B, Palacka K, Kossl J, **Bohacova P**, Krulova M, Brejchova J, Svoboda P, Zajicova A. The altered migration and distribution of systemically administered mesenchymal stem cells in morphine-treated recipients. Stem Cell Rev Rep. 2021;17:1420-1428.

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I hereby confirm that the author of this thesis, Pavla Boháčová, has contributed to the publications included in this thesis as it is described in section Aspirant´s contribution in subchapters of Results. She performed most of the experimental work and prepared manuscripts in case of her first´s author publications.

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prof. RNDr. Vladimír Holáň, DrSc.

4. Results

4.1. MSCs attenuate the adverse effects of immunosuppressive drugs on distinct T cell subpopulations

Michaela Hajkova, Barbora Hermankova, Eliska Javorkova, **Pavla Bohacova**, Alena Zajicova, Vladimir Holan, Magdalena Krulova
Stem Cell Rev Rep. 2017a;13:104-115.

The treatment combining cell-based therapy of MSCs with immunosuppressive drug application belongs among promising therapeutic strategies to reduce immunosuppressant doses, reduce their adverse effects, and improve their action. In the present study, we tested the effects of cyclosporine A, mycophenolate mofetil, rapamycin, prednisone, and dexamethasone in combination with MSCs on T cells.

The observations showed that usage of MSCs protected T cells from the proapoptotic effect of immunosuppressants and significantly reduced the expression of the activation marker, CD25 molecule, on T cells. It appeared that although the action of MSCs on T cells under the condition of immunosuppressive agents prevented T cell apoptosis, MSCs reduced the proportion of T cells with activated phenotype. The results varied according to immunosuppressant type, but generally, the effect of MSCs on T cells treated by immunosuppressants caused a reduction of T cell differentiation into effector Th17 ($ROR\gamma t^{+}$), Th2 ($GATA\text{-}3^{+}$) and Th1 ($T\text{-}bet^{+}$) cells, on the contrary, the proportion of Treg ($FoxP3^{+}$) cells was increased.

On the other hand, the production of lineage-specific cytokines (IL-17, IL-4, IFN-γ, and IL-10) did not directly correspond to the changes in the expression of lineage-specific transcription factors. These differences may be caused by the ability of immunosuppressive agents to suppress T cell proliferation but not to affect their activation simultaneously. MSC-based therapy can be a powerful tool to decrease immunosuppressive drug doses and their adverse effects in clinical practice. Nevertheless, the interactions of MSCs, immunosuppressive drugs, and the immune system are very complex and need future research.

Aspirant's contribution: participation in flow cytometry analyses, data analyses, and interpretation.

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Mesenchymal Stem Cells Attenuate the Adverse Effects of Immunosuppressive Drugs on Distinct T Cell Subopulations

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Abstract Immunosuppressive drugs are widely used to treat undesirable immune reaction, however their clinical use is often limited by harmful side effects. The combined application of immunosuppressive agents with mesenchymal stem cells (MSCs) offers a promising alternative approach that enables the reduction of immunosuppressive agent doses and simultaneously maintains or improves the outcome of therapy. The present study aimed to determinate the effects of immunosuppressants on individual T cell subpopulations and to investigate the efficacy of MSC-based treatment combined with immunosuppressive drugs. We tested the effect of five widely used immunosuppressants with different action mechanisms: cyclosporine A, mycophenolate mofetil, rapamycin, and two glucocorticoids - prednisone and dexamethasone in combination with MSCs on mouse $CD4^+$ and $CD8^+$ lymphocyte viability and activation, Th17 ($ROR\gamma t^+$), Th1 ($T\text{-}bet^+$), Th2 ($GATA\text{-}3^+$) and Treg ($Foxp3^+$) cell proportion and on the production of corresponding key cytokines (IL-17, IFNγ, IL-4 and IL-10). We showed that MSCs modulate the actions of immunosuppressants and in combination with immunosuppressive drugs display distinct effect on cell activation and balance among different T lymphocytes subpopulations and exert a suppressive effect on proinflammatory T cell subsets while promoting the functions of anti-inflammatory Treg lymphocytes. The results indicated that MSC-based therapy could be a powerful strategy to attenuate the negative effects of immunosuppressive drugs on the immune system.

Keywords Mesenchymal stem cells · Immunosuppressive drugs · Stem cell therapy · T cells · Immunomodulation

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Introduction

Immunosuppressive drugs are widely used to treat autoimmune or inflammatory diseases and to prevent the rejection of transplanted organs or tissues. However, their impact is often accompanied by unwanted harmful side effects such as nephrotoxicity or osteoporosis, which may diminish their overall benefits [1]. The combined application of immunosuppressive agents with mesenchymal stem cells (MSCs)-based therapy offers a promising alternative strategy enabling immunosuppressive drug doses to be reduced or improving their efficacy.

MSCs represent a heterogenous population of multipotent stem cells, which can be isolated from different tissues including heart, spleen, bone marrow, umbilical cord blood or adipose tissue [2, 3]. One of crucial characteristics of MSCs is their ability to regulate immune response. It has been well documented that MSCs inhibit lymphocyte proliferation, suppress production of proinflammatory cytokines and alter the balance of Th1/Th2/Th17/Treg lymphocytes [4–6]. In addition, the secretion of various growth and trophic factors [7] enables them to support tissue regeneration [8, 9], inhibit apoptosis and exert a cytoprotective effect [10, 11]. Together with their low immunogenicity, MSCs provide promising features for their use in treating many harmful immune reactions [12–14].

There are a few reports describing the potential therapeutic effect of MSCs used in combination with immunosuppressive

drugs that yielded inconsistent results. In an in vitro model of allogeneic response mycophenolate moefetil (MMF) promoted the inhibitory effect of MSCs; cyclosporine A (CsA), tacrolimus and rapamycin (Rapa) antagonized it and dexamethasone (Dex) had no effect [15]. However, Hoogduijn et al. [16] demonstrated that preincubation of MSCs with calcineurin inhibitors increased the efficacy of MSCs to regulate peripheral blood mononuclear cells proliferation whereas MMF or Rapa had no effect. Moreover, they pointed out the risk of toxicity of high doses of some immunosuppressants on MSCs. On the contrary, Chen et al. [17] showed that preincubation with CsA resulted in inhibition of MSC apoptosis. Therapy combining MSCs and different immunosuppressive drugs has been tested in in vivo animal models. It has been described that the combined application of MMF and MSCs leads to prolonged allograft survival [18, 19] and promising results were also obtained after application of MSCs with Rapa [20, 21]. In our recent report we showed, that the combination of MSCs and CsA led to a super-additive immunosuppressive effect in a model of skin allograft transplantation [22].

It has been shown that MSCs in combination with immunosuppressive drugs provide further potential benefits in the treatment of several disorders and are a perspective option for cell therapy [23–25]. MSCs are now used in more than 500 registered clinical trials (http://clinicaltrials.gov/). However, so far, the mechanisms of MSC interaction with different immunosuppressive drugs and how they affect the treatment of inflammatory reaction remains unclear. In this study, we aimed to investigate influence of MSCs on the effects of clinically relevant doses [15, 26] of five immunosuppressive drugs with different mechanisms of action (calcineurin inhibitor - CsA, mTOR inhibitor - Rapa, inosine monophosphate dehydrogenase inhibitor - MMF and two steroids - Dex and prednisone (Pred)). We demonstrated that MSCs in combination with immunosuppressive drugs modulate the adverse effects of immunosuppressive agents on T cells, direct differentiation of naïve T cells toward distinct functional subsets of T lymphocytes (Th17, Th1, Th2, Tc, Treg) and regulate intracellular cytokine expression by these populations. Our study suggest that application of MSCs affects the therapeutic effectiveness of immunosuppressive medication. These findings are important for the potential use of MSCs in combination with immunosuppressive drugs in clinical practice.

Materials and Methods

Mice

Female BALB/c mice at the age of 8–12 weeks were obtained from the breeding unit of the Institute of Molecular Genetics, Prague, Czech Republic. The protocol and use of animals was approved by the local Animal Ethics Committee.

Isolation and Culture of MSCs

The inguinal fat pads were harvested from BALB/c mice, washed in phosphate buffered saline (PBS), cut into small pieces with scissors and digested with a 1% solution of collagenase I (Sigma, St. Louis, MO, USA) in PBS for 60 min at 37°C with gentle agitation. The cell suspension was washed in PBS and centrifuged (250 g, 8 min). The washing step was repeated 3 times. The pellet was resuspended and cultured in Dulbecco's modified Eagle medium (DMEM, PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum (FCS; Sigma), antibiotics (100 μg/ml of streptomycin, 100 U/ml of penicillin) and 10 mM Hepes buffer (hereinafter referred to as complete DMEM) in 75 cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere of 5% CO_2. After a 48-h incubation, the nonadherent cells were removed by washing and the remaining adherent cells were cultured with regular exchange of the culture medium and passaging of the cells to maintain an optimal cell concentration. Cells between passage 3 and 5 were used.

Immunosupressive Drugs

Cyclosporine (CsA), mycophenolate mofetil (MMF), rapamycin (Rapa), dexamethasone (Dex) and prednisone (Pred) (all purchased from Sigma) were dissolved in dimethyl sulfoxide (DMSO) in concentration 5 mg/ml and stored at −20°C. In each experiment a vehicle control was included. Corresponding concentration of DMSO had no effects on cell activities (data not shown).

Characterisation of MSCs by Flow Cytometry

MSCs were incubated without or with immunosuppressive drugs (CsA, MMF, Rapa, Pred and Dex at concentrations 5, 0.5, 0.05 and 0.005 μg/ml) for 48 h, washed in PBS containing 0.5% bovine serum albumine (BSA) and then incubated for 30 min on ice with the following anti-mouse monoclonal antibodies (mAb): allophycocyanine (APC)-labelled anti-CD44 (clone IM7, BD Pharmingen, San Jose, CA, USA), fluorescein isothiocyanate (FITC)-labelled anti-CD90.2 (clone 30-H12, BioLegend, San Diego, CA, USA), phycoerythrin (PE)-labelled anti-CD105 (clone MJ/18, eBioscience, San Diego, CA, USA), APC-labelled anti-CD11b (clone M1/70, BioLegend), PE-labelled anti-CD31 (clone MEC 13.3, BD Pharmingen) or PE-labelled anti-CD45 (clone 30-F11, BioLegend). Dead cells were stained using Hoechst 33,258 fluorescent dye (Sigma) added to the samples 15 min before flow cytometry analysis. Data were collected using a LSRII cytometer (BD Bioscience, Franklin Lakes, NJ, USA) and analysed using Gatelogic 400.2A software (Invai, Mentone, Australia).

Determination of MSC Proliferation and Viability

The metabolic activity of living cells was determined by the WST assay. MSCs (0.03×10^6/ml) were incubated alone or with immunosuppressive drugs at a concentration of 5, 0.5, 0.05 and 0.005 μg/ml in a volume of 0.2 ml of complete DMEM in 96-well tissue culture plates (Corning Co., Corning, NY, USA) for 48 h at 37°C in a humidified atmosphere of 5% CO_2. WST-1 reagent (Roche, Mannheim, Germany) (1:10 final dilution) was added to each well, and the plates were incubated for another 2 h to form formazan. The absorbance was measured using an ELx800 ELISA Reader (BioTek, Bad Friedrichshall, Germany) at a wavelength of 450 nm.

Regulation of Spleen Cell Proliferation

Spleen cells (0.75×10^6 cells/ml) were stimulated in a volume of 0.2 ml of RPMI 1640 medium (Sigma) supplemented with 10% FCS, antibiotics (penicillin, streptomycin), 10 mM Hepes buffer and 5×10^{-5} M 2-mercaptoethanol (hereinafter a complete RPMI 1640 medium) in 72-well tissue culture plates (Corning) with Concanavaline A (ConA, 1.25 μg/ml, Sigma) and with immunosuppressive drugs at a concentration of 5; 0.5, 0.05 and 0.005 μg/ml. Cell proliferation was determined by adding ^{3}H–thymidine (1 μCi/well, PE systems, Walthan, MA, USA) for the last 6 h of the 96-h incubation period.

Cytokine Production and Detection

To test the production of interleukin 2 (IL-2) and interferon γ (IFNγ), spleen cells (1.25×10^6/ml) were incubated in 48-well tissue culture plates (Nunc) in a final volume of 0.6 ml of complete RPMI 1640 medium stimulated with ConA (1.25 μg/ml) for 24 (IL-2) or 48 (IFNγ) h. To test the effect immunosuppressive drugs on spleen cells, CsA, MMF, Rapa, Pred and Dex (5, 0.5, 0.05 and 0.005 μg/ml) were added to the cultures. The production of IL-2 and IFNγ was determined by an enzyme-linked immunosorbent assay (ELISA) using cytokine-specific capture and detection mAbs purchased from BD Pharmingen (San Diego, CA, USA) and following the manufacturer's instructions. The reactions were quantified by spectrophotometry using an ELx800 ELISA Reader.

Co-Cultures of MSCs and Lymphocytes

Spleen cells (1.5×10^6/ml) were incubated in 24-well tissue culture plates (Nunc) in a final volume of 1 ml of complete RPMI 1640 medium stimulated with ConA (1.25 μg/ml) for 48 h (to determine apoptosis, T lymphocyte activation and intracellular detection of cytokines) or 72 h (for intracellular detection of RORγt, T-bet, Foxp3 and GATA-3). To test the effect of MSCs in combination with immunosuppressive drugs on spleen cells, MSCs at the ratio 1:30 and CsA (0.05, 0.005 μg/ml), MMF (0.5, 0.05 μg/ml), Rapa (0.05, 0.005 μg/ml), Pred (0.5, 0.05 μg/ml) and Dex (0.5, 0.05 μg/ml) were added to the cultures.

Detection of Apoptosis

Spleen cells cultured for 48 h with MSCs and immunosuppressive drugs were harvested, washed in PBS containing 0.5% BSA and were incubated for 30 min on ice with FITC-labelled anti-CD3 mAb (clone 17A2, BioLegend), FITC-labelled anti-CD4 mAb (clone KG1.5, BioLegend), FITC-labelled anti-CD8a mAb (clone53–6.7, BioLegend) and PE-labelled anti-CD45 mAb (clone 30-F11, BioLegend). After washing in PBS containing 0.5% BSA, cells were stained for Annexin V using an Annexin V apoptosis detection kit according to the manufacturer's protocol (Apronex, Jesenice, Czech Republic). Dead cells were discriminated using Hoechst 33258 fluorescent dye (Sigma) added to the samples 15 min before flow cytometry analysis. Data were collected using a LSRII cytometer (BD Bioscience) and analysed using Gatelogic 400.2A software (Invai).

Regulation of T Lymphocyte Activation

To test the effect of MSCs in combination with immunosuppressive drugs on T lymphocyte activation, the cultured spleen cells were harvested, washed in PBS containing 0.5% BSA and stained for 30 min on ice with FITC-labelled mAb anti-CD4 (clone GK1.5, BD Pharmingen), APC-labelled mAb anti-CD8a (clone 53–6.7, BioLegend), FITC-labelled mAb anti-CD25 (clone 3C7, BioLegend), APC-labelled mAb anti-CD25 (clone PC61, BioLegend) and PE-labelled mAb anti-CD45 (clone 30-F11, BioLegend). Dead cells were stained using Hoechst 33,258 fluorescent dye (Sigma) added to the samples 15 min before flow cytometry analysis. Data were collected using a LSRII cytometer (BD Bioscience) and analysed using Gatelogic 400.2A software (Invai, Mentone, Australia).

Intracellular Staining of RORγt, T-bet, GATA-3 and Foxp3

The cultured spleen cells were harvested and washed with PBS containing 0.5% BSA. Before intracellular staining cells were incubated for 30 min on ice with FITC-labelled mAb anti-CD4 (clone GK1.5, BD Pharmingen), Alexa Fluor 700-labelled mAb anti-CD45 (clone 30-F11, BioLegend) and Live/Dead Fixable Violet Dead Cell Stain Kit (Molecular Probes, Eugene, OR, USA) for staining dead cells. Cells were washed in PBS containing 0.5% BSA, fixed and permeabilized using a Foxp3 Staining Buffer Set (eBioscience)

according to the manufacturer's instructions. For intracellular detection of RORγt, T-bet, GATA-3 and Foxp3, the cells were stained for 30 min with PE-labelled mAb anti-RORγt (clone AFKJS-9, eBioscience), APC-labelled mAb anti-T-bet (clone eBio4B10, eBioscience) PE-labelled mAb anti-GATA-3 (clone TWAJ, eBioscience) and PE-labelled mAb anti-Foxp3 (cloneFJK-16 s, eBioscience). Data were collected using a LSRII cytometer (BD Bioscience) and analysed using Gatelogic 400.2A software (Invai).

Intracellular Detection of IL-4, IL-10, IL-17 and IFNγ

To analyse the intracellular IL-4, IL-10, IL-17 and IFNγ expression, phorbol myristate acetate (PMA; 20 ng/ml, Sigma), ionomycin (1 μg/ml, Sigma) and Brefeldin A (5 μg/ml, eBioscience) were added to the cultures for the last 5 h of the 48-h incubation period. Cells were than harvested, washed with PBS containing 0.5% BSA and were incubated for 30 min on ice with FITC-labelled mAb anti-CD4 (clone GK1.5, BD Pharmingen), APC-labelled mAb anti-CD4 (clone GK1.5, BioLegend), APC-labelled mAb anti-CD8a (clone 53–6.7, BioLegend), Alexa Fluor 700-labelled mAb anti-CD45 (clone30-F11, BioLegend) and Live/Dead Fixable Violet Dead Stain Kit (Molecular Probes) for staining dead cells before intracellular staining. A Fixation and Permeabilization Kit (eBioscience) was used for permeabilization according to the manufacturer's instructions. The cells were intracellularly stained for 30 min with PE-labelled mAb anti-IL-4 (clone 11B11, eBioscience), APC-labelled mAb anti-IL-10 (clone JES5-16E3, eBioscience), PE-labelled mAb anti-IL-17 (clone eBio17B7, eBioscience) and FITC-labelled mAb anti-IFNγ (clone XMG1.2, eBioscience). Data were collected using a LSRII cytometer (BD Bioscience) and analysed using Gatelogic 400.2A software (Invai).

Statistical Analysis

The Prism 5 program (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The results are expressed as the mean ± standard error (SE). The statistical significance of differences between the means of individual groups was calculated using one way analysis of variance (ANOVA). *P* values less than 0.05 were considered statistically significant.

Results

Immunosuppressive Drugs Affect the Metabolic Activity of MSCs but not their Surface Marker Expression

Adipose tissue-derived MSCs growing for 3 weeks in culture were phenotypically characterized by flow cytometry. As demonstrated in Fig. 1a, untreated and immunosuppressive drug-treated MSCs were negative with a corresponding intensity for CD11b, CD31 and CD45 markers and were positive for CD44, CD90.2 and CD105, which are markers attributed to murine MSCs, in each concentration of immunosuppressive drugs used (only results for the highest used concentration of immunosuppressive drugs are shown). After culture expansion, MSCs showed a typical spindle-shaped fibroblastic morphology. Under appropriate culture conditions, both untreated and immunosuppressive drug-treated MSCs were able to differentiate into adipocytes or osteoblasts (data not shown).

To further characterise the effects of immunosuppressive drugs on MSCs, the cells were treated with increasing concentrations of CsA, MMF, Rapa, Pred and Dex and their proliferation and viability was determined by the WST assay. As demonstrated in Fig. 1b, MSCs are susceptible to Rapa and to the highest concentration of MMF. All the other samples had comparable metabolic activity.

Immunosuppressive Drugs Inhibit Spleen Cells Proliferation and Production of IL-2 and IFNγ in a Dose-Dependent Manner

To select the optimal concentrations of immunosuppressive drugs that inhibit lymphocyte activation, but not MSCs viability, the effect of used drugs on splenocyte proliferation was tested. As demonstrated in Fig. 2a, all tested drugs inhibited in a dose dependent manner proliferation of ConA-stimulated lymphocytes.

Next, we evaluated the effects of immunosuppressive drugs on the production of IL-2 (Fig. 2b) and IFNγ (Fig. 2c), by ConA stimulated splenocytes. Similar to the effect observed for proliferation, the highest concentrations of immunosuppressants significantly inhibited the production of these cytokines, while at the lowest concentration, the capacity to produce IL-2 and IFNγ was partially restored.

On the basis of the above results from dose-response assays for MSCs and splenocytes, two concentrations (CsA 0.5 and 0.005, MMF 0.5 and 0.05, Rapa 0.05 and 0.005, Pred 0.5 and 0.05, Dex 0.5 and 0.05 μg/ml) of each immunosuppressant were selected for following experiments.

MSCs Improve T Lymphocytes Viability

The effect of immunosuppressants and their combination with MSCs on $CD3^+$ T cell apoptosis was tested by Annexin V staining. The presence of Annexin V^+ Hoechst 33258^- (apoptotic) and Annexin V^+ Hoechst 33258^+ (dead) cells increased after treatment with all immunosuppressive drugs (Fig. 3a). A higher percentage of apoptotic cells was observed

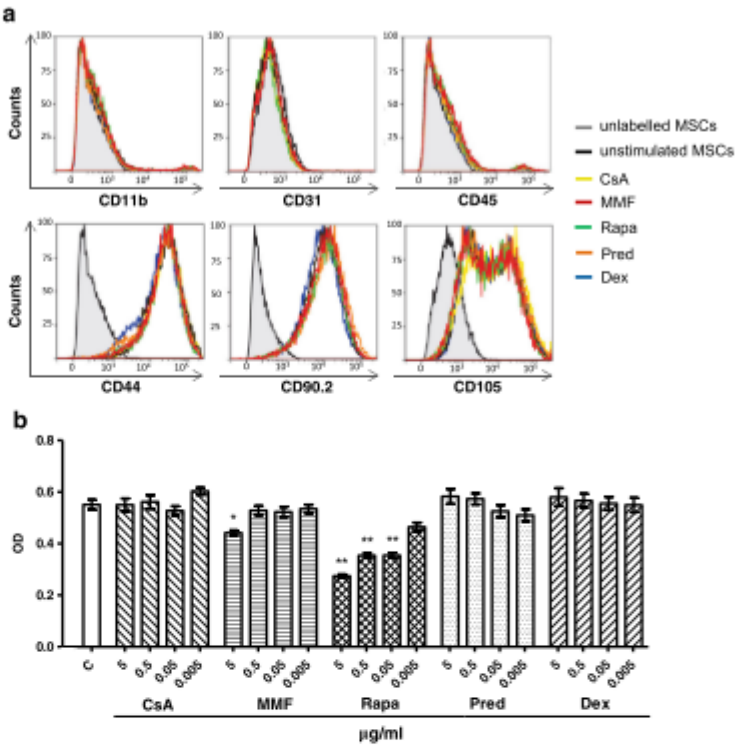

Fig. 1 The effect of immunosuppressive drugs on the phenotype and metabolic activity of MSCs. Flow cytometry analysis did not show significant changes in the expression of CD11b, CD31, CD44, CD45, CD90.2 and CD105 MSCs treated with CsA, MMF, Rapa, Pred and Dex in comparison with untreated MSCs (**a**). One representative of 3 independent experiments is presented. The metabolic activity of untreated or immunosuppressive drug treated MSCs was evaluated by WST assay (**b**). Data are expressed as the means ± SE from five independent experiments. Groups with asterisks are significantly different (*$p < 0.05$, **$p < 0.01$) from control (untreated MSCs, C)

in samples treated with CsA, Pred and Dex. However, this effect of immunosuppressants on T lymphocyte viability was partially moderated by MSCs. Accordingly with this observation, the proportion of live lymphocytes significantly increased in samples treated with MSCs. The changes in apoptosis induction are similar for both $CD4^+$ (Fig. 3b) and $CD8^+$ (Fig.3c) lymphocytes.

MSCs Downregulate CD25 Expression on Immunosuppressive Drug Treated T Lymphocytes

MSCs were tested for their capability to inhibit activation of ConA-stimulated spleen cells. Spleen cells were stimulated with ConA and cultured for 48 h in the presence or absence of MSCs and immunosuppressive drugs and the percentage of activated ($CD25^+$) T cells was determined. As shown in Fig. 4a, MSCs suppressed activation of $CD4^+$ lymphocytes with the greatest decline in groups treated with Pred and Dex (Fig. 4a). This effect was even more pronounced on $CD8^+$ lymphocyte activation (Fig. 4b).

MSCs in Combination with Immunosuppressive Drugs Regulate the RORγt, T-bet, GATA-3 and Foxp3 Expression

To further analyse the effect of the treatment on individual T cell populations, splenocytes were stimulated for 72 h with ConA in the presence or absence of MSCs and immunosuppressive drugs and the percentage of $CD4^+RORγt^+$ (Th17), $CD4^+$T-bet^+ (Th1) $CD4^+GATA\text{-}3^+$ (Th2) and $CD4^+Foxp3^+$ (Treg) cells was determined. As demonstrated in Fig. 5a, MSCs significantly suppressed RORγt expression in the majority of treated groups. This effect was most evident in groups treated with 0.05 μg/ml MMF, Pred and Dex, where the drug alone did not decrease the percentage of Th17 cells. The immunosuppressants, with the exception of MMF, significantly decreased T-bet expression, as in case of RORγt, the presence of MSCs in cultures further downregulated the percentage of T-bet positive cells in all samples (Fig. 5b). Figure 5c demonstrates, that the presence of MSCs in the cultures containing immunosuppressants significantly decreased the proportion of $CD4^+GATA\text{-}3^+$ cells in all groups. As shown in Fig. 5d, the

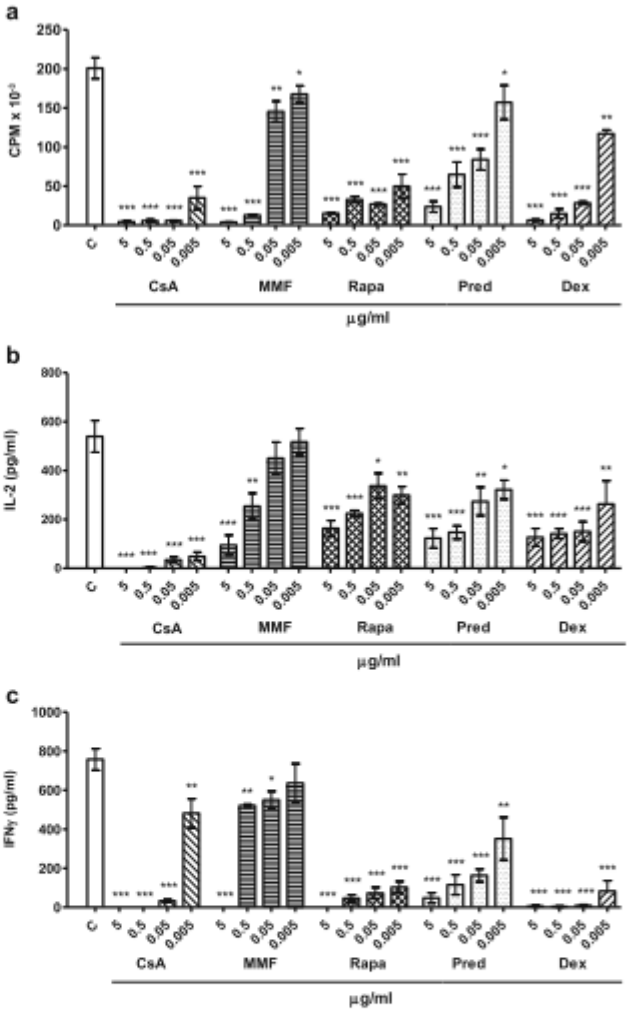

Fig. 2 Immunosuppressive drugs modulate T lymphocytes activity in a dose-dependent manner. Spleen cells were stimulated with ConA in the absence or presence of immunosuppressive drugs and the lymphocytes proliferation was determined after a 72-h incubation (**a**). Production of IL-2 (**b**) and IFNγ (**c**) by spleen cells stimulated with ConA and treated with immunosuppressants was determined by ELISA after a 24-h (IL-2) or 48-h (IFNγ) incubation. Data are expressed as the means ± SE from five independent experiments. Groups with asterisks are significantly different (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) from control (untreated splenocytes, C)

proportion of $CD4^+Foxp3^+$ Treg cells was augmented in the presence of MSCs. The most apparent increase in the number of $Foxp3^+$ cells was observed in groups treated with MSCs in combination with CsA, Rapa and Dex.

MSCs in Combination with Immunosuppressants Modulate IL-4, IL-10, IL-17 and IFN-γ Production

To provide direct evidence that MSCs in combination with immunosuppressive drugs also modulate the functions of different T cells populations, spleen cells were stimulated with ConA for 48 h in the presence or absence of immunosuppressive drugs and MSCs. The intracellular level of cytokines specific for individual T cell subsets (IL-17, IFNγ, IL-4 and IL-10 in $CD4^+$ cells and IFN-γ in $CD8^+$ cells) were determined. As shown in Fig. 6a, treatment with immunosuppressants did not significantly attenuate the proportion of $CD4^+IL\text{-}17^+$ cells, rather, we observed upregulation in samples treated with Pred and Dex. However, MSCs completely abrogated this effect and significantly downregulate the expression of IL-17 in the presence of all tested drugs. As shown in Fig. 6b, the percentage of $CD4^+IFN\gamma^+$ cells significantly

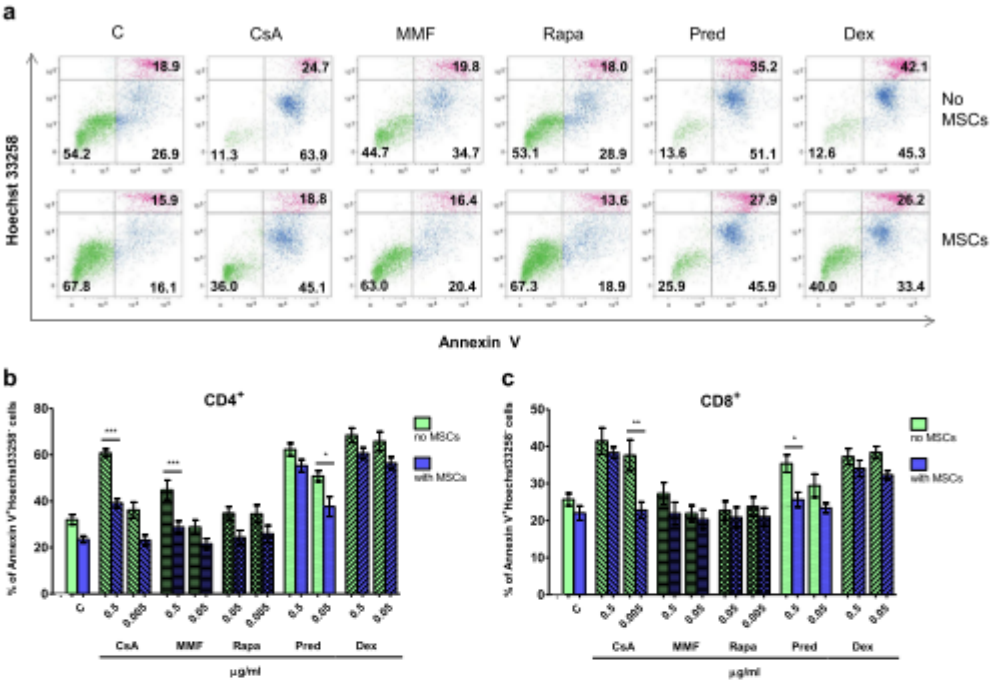

Fig. 3 The effect of immunosuppressive drugs and MSCs on splenocyte viability. Spleen cells were stimulated with ConA in the absence or presence of MSCs and immunosuppressive drugs for 48 h. The number of live (Annexin V$^-$Hoechst 33258$^-$), apoptotic (Annexin V$^+$Hoechst33258$^-$) and dead (Hoechst 33,258$^+$Annexin V$^+$) CD3$^+$ T cells was determined by flow cytometry (**a**). One representative of 3 independent experiments is presented. The effects of immunosuppressive drugs on CD4$^+$ (**b**) and CD8$^+$ (**c**) cells were further studied. Data are expressed as the means ± SE from five independent experiments. Statistical significance between groups is marked with asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$), (untreated cells, C)

decreased after treatment with the majority of used drugs and this effect was more evident in the presence of MSCs. We observed downregulation of the CD4$^+$IL-4$^+$ population in the presence of the majority of drugs with an even more apparent decrease in the groups treated with MSCs (Fig. 6c). Figure 6d demonstrates that similar to CD4$^+$IFNγ$^+$ cells, the frequency of the CD8$^+$IFNγ$^+$ cells was significantly suppressed in the presence of MSCs. In contrast, the percentage of CD4$^+$IL-10$^+$ cells stayed similar in all groups with the tendency of upregulation observed in cultures treated with MMF or Dex in combination with MSCs. (Fig. 6e).

Discussion

In several pathological conditions the suppression of harmful immune reaction is the main issue. Treatment combining transplantation of MSCs with immunosuppressive drug application ranks among the very promising new therapeutic strategies aiming to reduce immunosuppressant doses, eliminate their adverse effects and to improve their action. Although the suppressive effects of MSCs cultured in combination with different immunosuppressants on T lymphocyte proliferation have been described [15, 16], a limited knowledge is available about modulatory action on the individual T-cell subsets. Our study was specifically designed to examine the interactions between MSCs and immunosuppressive drugs in vitro and to determine how such treatment modulated the balance among individual T lymphocyte subpopulations and whether it affected their function.

Untreated or drug-treated MSCs showed no significant changes in the expression of the surface markers associated with murine MSCs (CD44, CD90.2 and CD105) after cultivation with tested immunosuppressants. However, high concentrations of MMF and Rapa significantly downregulate MSC proliferation and viability. A similar observation has been reported by Hoogdujien et al. [16], who tested the effect of Tacrolimus, MMF and Rapa on human MSC proliferation.

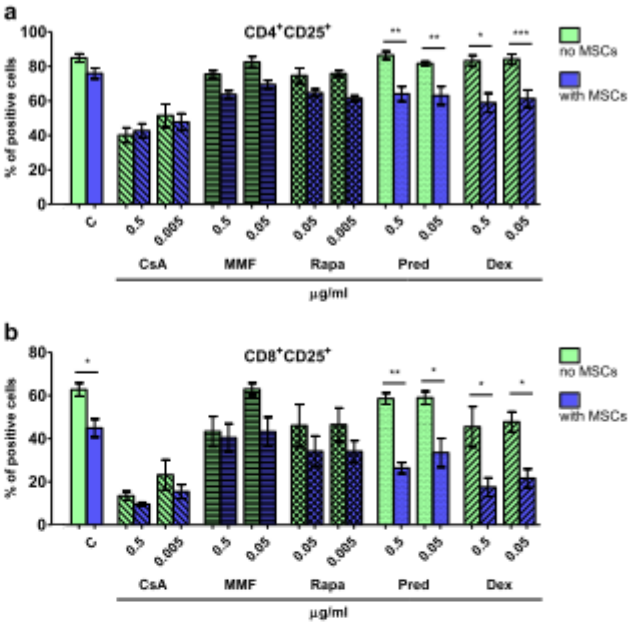

Fig. 4 The effect of MSCs and immunosuppressive drugs on the expression of activation marker CD25 on T cells. Spleen cells were stimulated with ConA in the absence or presence of MSCs and immunosuppressive drugs for 48 h. Flow cytometry analysis showed that MSCs downregulate the percentage of $CD4^+CD25^+$ (**a**) as well as $CD8^+CD25^+$ (**b**) cells. Data are expressed as the means ± SE from five independent experiments. Statistical significance between groups is marked with asterisks ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$), (untreated cells, C)

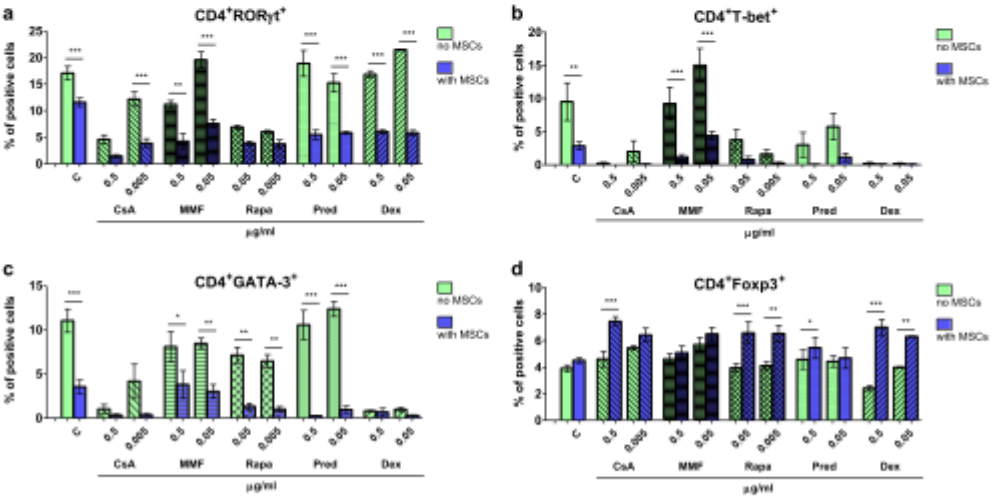

Fig. 5 Regulation of RORγt, T-bet, Foxp3 and GATA-3 expression by immunosuppressive drugs and MSCs. Spleen cells were stimulated with ConA in the absence or presence of MSCs and immunosuppressive drugs for 72 h and analysed using flow cytometry. The treatment reduces the percentage of $CD4^+RORγt^+$ (**a**), $CD4^+T\text{-}bet^+$ (**b**) and $CD4^+GATA\text{-}3^+$ (**c**) populations in all groups. On the contrary, the proportion of $CD4^+Foxp3^+$ Treg (**d**) was slightly upregulated. Data are expressed as the means ± SE from five independent experiments. Statistical significance between groups is marked with asterisks ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$), (untreated cells, C)

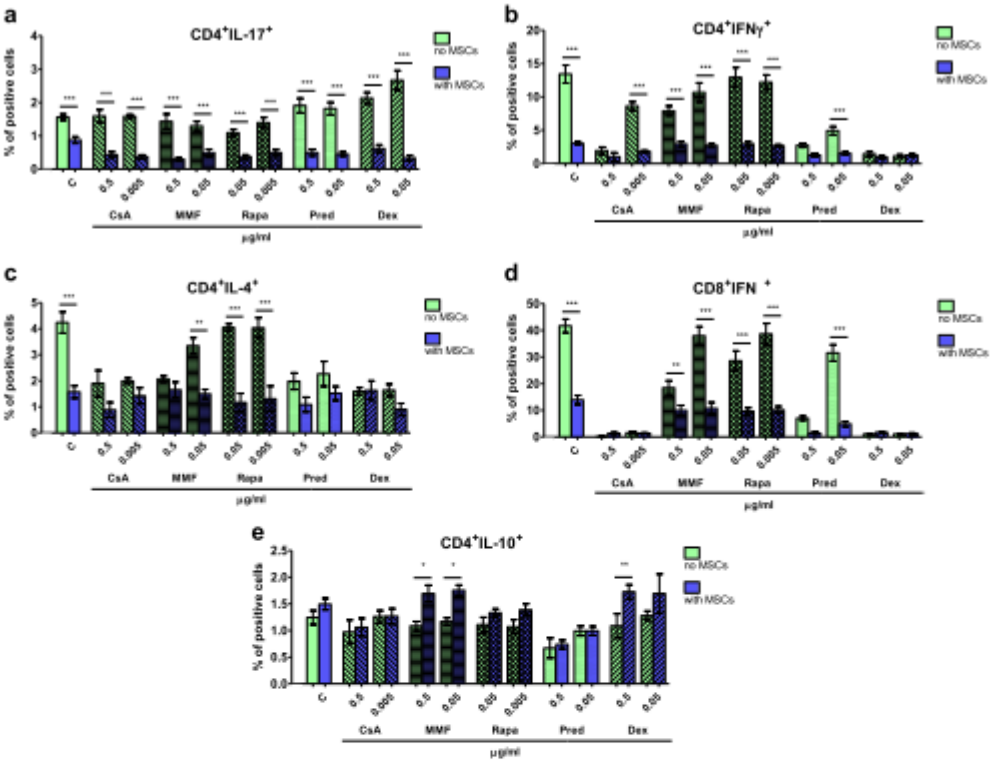

Fig. 6 The effect of MSCs and immunosuppressive drugs on IL-17, IFN-γ, IL-10 and IL-4 producing cells. Spleen cells were stimulated for 48 h with ConA in the absence or presence of immunosuppressive drugs and MSCs. The flow cytometry analysis showed a significant downregulation of the proportion of $CD4^+IL\text{-}17^+$ (**a**), $CD4^+IFN\text{-}\gamma^+$ (**b**), $CD4^+IL\text{-}4^+$ (**c**) and $CD8^+IFN\gamma^+$ (**d**) cells. In addition the preservation or upregulation of $CD4^+IL\text{-}10^+$ (**e**) cells was observed. Data are expressed as the means ± SE from five independent experiments. Statistical significance between groups is marked with asterisks ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$), (untreated cells, C)

To select the optimal concentrations of drugs for the further study, we performed a dose-response tests, and the concentrations which significantly suppressed T lymphocyte functions, but did not decrease viability of MSCs, were used for further experiments.

Induction of apoptosis is a key mechanism to delete activated T cells in the periphery during the termination of an immune response [27], but subsequent deficits of immune cells can lead to the development of opportunistic infections or malignancies [28]. Our results showed that the treatment of spleen cells with immunosuppressive drugs increases the proportion of apoptotic and dead $CD3^+$ T cells and at the same time decreases the percentage of living cells in the culture. It has been described that MSCs inhibit the spontaneous death of lymphocytes [29] and are able to inhibit proliferation of activated T cells while simultaneously supporting their survival [30, 31]. We found that the presence of MSCs in cultures partially rescued cells from the apoptotic pathway induced by immunosuppressants. Furthermore our results showed that the above mentioned effects are similar for both $CD4^+$ and $CD8^+$ T cell populations.

MSCs possess the ability to inhibit the expression of activation marker CD25 on Th and Tc lymphocytes [32] and our results demonstrated that this downregulation could be further intensified by the presence of immunosuppressants. Taken together, it appears that although MSCs rescued $CD4^+$ and $CD8^+$ lymphocytes from apoptosis caused by immunosuppressive agents, they promoted the inhibitory action of immunosuppressants by reducing the proportion of cells with activated phenotype.

So far, disregulation of composition of various T cell subpopulations and their related cytokines has been proposed to

play a crucial role as an inflammation-promoting mechanism in autoimmune diseases or transplantation reaction [33, 34]. It has been well documented that MSCs possess the ability to modulate this balance [5, 6, 35]. Our results demonstrated that treatment combining MSCs and immunosuppressants significantly decreased the proportion of $CD4^+ROR\gamma t^+$ Th17 cells as well as the percentage of the IL-17^+ cells and thus the functional properties of these cells. Moreover, we found a synergistic effect of immunosuppressive agents and MSCs. Intriguingly, Pred and Dex showed a tendency to upregulate RORγt and IL-17 expression. These findings confirmed the observation of Prado et al. [36], who pointed out that glucocorticoids rather activated Th17 cells and caused Th17/Th1 imbalance in patients with systemic lupus erythematosus (SLE). On the other hand MMF inhibits Th17 related immune response [37] and thus MMF is considered a suitable drug for use in transplantation medicine [19, 38]. However, our results have shown that lower concentrations of MMF slightly upregulated RORγt expression. These inconsistent results might be due to differences in the drug metabolism in vivo and in an in vitro systems. Nevertheless, the stimulatory effect of both MMF and glucocorticoids on Th17 cells was completely abrogated by MSCs and the expression of RORγt and IL-17 was significantly decreased in the presence of MSCs. As with Th17 cells, the percentage of $CD4^+$T-bet^+ cells was decreased in the presence of immunosuppressive agents with the exception of MMF. However, also in this group the expression of T-bet cells was decreased in the presence of MSCs. We observed a significant decrease in the percentage of both $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ populations after co-culture with MSCs with the most significant suppression in the CsA- and Dex-treated groups.

It has been shown that depending on specific conditions during the development and progression of different diseases, MSCs are able to either promote [39] or downregulate [40] Th2 related immune response. In the present study, we observed that MSCs had the ability to supress both GATA-3 and IL-4 expression in all immunosuppressive drug treated samples. This might be beneficial especially in the treatment of Th2 mediated inflammatory diseases, such as allergic asthma or SLE. In contrast to the downregulation of Th17, Th1, Th2 and Tc cells, MSCs in combination with immunosuppressive agents increased the Treg proportion in all samples. Furthermore, we showed that MSCs promoted IL-10 expression and thus supported the regulatory function of $CD4^+$ cells. A significant increase of the $CD4^+IL\text{-}10^+$ population was observed in cultures combining treatment with MMF or a higher concentration of Dex with MSCs. Interestingly, we found that the changes in the expression of transcription factors of distinct T lymphocytes populations did not correlate with the changes in production of population related cytokines. These differences might be due to ability of immunosuppressive drugs to inhibit T lymphocyte proliferation but at the same time maintain their activity [41]. Moreover, it has been suggested by Prado et al. that upregulation of transcription factor expression in T cells after treatment is not necessarily associated with the increase of their cytokine production [36].

The presence of immunosuppressive drugs with different mechanisms of the action modulates the cytokine environment in the culture, which subsequently interacts with MSCs and affect their immunomodulatory action. Furthermore it has been shown that distinct T lymphocyte population display different sensitivity to immunosuppressive drugs [32, 42, 43] and individual drugs also interact with different molecular target in MSCs [16].

This study sheds new light on several reports indicating that MSCs in combination with immunosuppressive agents might be useful in preventing harmful immune response. We have shown that MSCs modulate the effect of immunosuppressive drugs and that therapy combining immunosuppressive agents with MSCs could favourably influence immune balance by harnessing the Th17/Th1/Th2/Tc related responses while preserving the anti-inflammatory Treg phenotype. In addition, these data clearly demonstrated that MSCs are also able to reverse the undesirable effects of immunosuppressive drugs such as the upregulation of the Th17 related response. Our data also underscored that the observed therapeutic benefits are not simply a result of the additive effects of MSCs and drugs, but that the interactions between MSCs, individual immunosuppressive drugs and the immune system are very complex. Understanding these mechanisms is essential for translation of MSCs in combination with immunosuppressive drugs into clinical practice.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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4.2. Distinct immunoregulatory mechanisms in MSCs: Role of the cytokine environment

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MSCs exert vast immunomodulatory potential, and the priming by cytokines influences their immunoregulatory activities. The complexity of MSC action mechanisms was studied in a model of reducing IL-10 production of B cells. We showed that IL-4 and IFN-γ, functionally distinct molecules, potentiated the ability of MSCs to suppress IL-10 production of LPS-stimulated B cells by different mechanisms.

We demonstrated that MSCs co-cultivated with LPS-stimulated B cells under IL-4 or IFN-γ conditions inhibited IL-10 production by these B cells. A previous study showed that the incubation or even preincubation of IFN-γ with MSCs led to the suppression of IL-10 production of B cells. This MSC attribution was dependent on cell-to-cell contact and involved the COX-2/PGE_2 signaling pathway. This effect was reversed using transwell cell-culture techniques or using cultivation with indomethacin, a COX-2 inhibitor. In the present study, observations revealed that IL-4 is another cytokine synergizing with MSCs in the suppression of IL-10 production of LPS-stimulated B cells. We tested the role of the COX-2 signaling cascade in IL-4-primed MSC inhibition of IL-10 production by B cells. The results showed that this inhibition was independent of the COX-2 enzyme and its products. The suppression of the IL-10 production of LPS-stimulated B cells by IL-4-primed MSCs also required cell-to-cell contact between MSCs and B cells. However, MSCs preincubated with IL-4 did not affect the IL-10 production of B cells contrary to MSCs pretreated with IFN-γ.

Priming of MSCs by cytokines can adjust their immunomodulatory properties. Different pro-inflammatory cytokines trigger various signaling pathways in MSCs. Despite signaling cascade, cytokine-primed MSCS can initiate a similar response of targeted cells, which highlights the complexity of MSC immunomodulatory properties.

Aspirant's contribution: flow cytometry analyses, data analyses, and interpretation.

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Distinct Immunoregulatory Mechanisms in Mesenchymal Stem Cells: Role of the Cytokine Environment

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Abstract Mesenchymal stem cells (MSCs) represent a population of cells which have the ability to regulate reactivity of T and B lymphocytes by multiple mechanisms. The immunoregulatory activities of MSCs are strictly influenced by the cytokine environment. Here we show that two functionally distinct cytokines, interleukin-4 (IL-4) and interferon-γ (IFN-γ), significantly potentiate the ability of MSCs to inhibit IL-10 production by activated regulatory B cells (Bregs). However, MSCs in the presence of IL-4 or IFN-γ inhibit the IL-10 production by different mechanisms. Preincubation of MSCs with IFN-γ led to the suppression, but pretreatment with IL-4 of neither MSCs nor B cells resulted in the suppression of IL-10 production. The search for candidate regulatory molecules expressed in cytokine-treated MSCs revealed different patterns of the gene expression. Pretreatment of MSCs with IFN-γ, but not with IL-4, induced expression of indoleamine-2,3-dioxygenase, cyclooxygenase-2 and programmed cell death-ligand 1. To identify the molecule(s) responsible for the suppression of IL-10 production, we used specific inhibitors of the putative regulatory molecules. We found that indomethacine, an inhibitor of cyclooxygenase-2 (Cox-2) activity, completely abrogated the inhibition of IL-10 production in cultures containing MSCs and IFN-γ, but had no effect on the suppression in cell cultures containing MSCs and IL-4. The results show that MSCs can inhibit the response of B cells to one stimulus by different mechanisms in dependence on the cytokine environment and thus support the idea of the complexity of immunoregulatory action of MSCs.

Keywords Mesenchymal stem cells · Regulatory B cells · Cytokine environment · IL-10 · IL-4 · IFN-γ · Cyclooxygenase 2 · Gene expression · Immunoregulation

Introduction

The final manifestation of immune response is a result of a complex interaction and cooperation of phenotypically and functionally different cell populations and cytokines. One of cell populations which modulate reactivity of cells of natural and adaptive immunity, are mesenchymal stem cells (MSCs). These cells can be found in nearly all tissues and organs and are characterized by the ability to differentiate into various cell types. In addition to their differentiation potential, MSCs are a potent source of a number of various cytokines and other growth factors and regulate immune response in both positive and negative manner. It has been shown that MSCs inhibit proliferation of T and B lymphocytes [1, 2] regulate production of cytokines [3, 4], modulate functions of antigen-presenting cells [5, 6] and suppress development of cytotoxic T and NK cells in vitro [7, 8]. MSCs also inhibit autoimmune, transplantation and inflammatory reactions in vivo [9–11]. For their immunomodulatory activity MSCs utilize multiple mechanisms which include secretion of regulatory cytokines and other factors, the expression of inhibitory membrane molecules, negative effects on antigen-presenting cells and activation of regulatory T cells [12] Although MSCs themselves are a potent source of cytokines and other immunoregulatory molecules, their activity is regulated by other cytokines. It has been shown that cytokine production and phenotypic profiles

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of MSCs change in dependence on cytokine environment [13, 14]. The complexity of immunoregulatory action of MSCs is supported by the observation that MSCs inhibited lymphocyte proliferation induced by mitogens and alloantigens by different mechanisms [15].

Although there are extensive published data on the effects of MSCs on T cells or antigen-presenting cells, considerably less knowledge is available about the effects of MSCs on B lymphocytes. In these studies, the suppression of B cell differentiation and inhibition of antibody production was demonstrated [16, 17]. Within the population of B cells, a subpopulation called regulatory B cells (Bregs) which is characterized by the ability to suppress immune reactions in antibody-independent manner, has been identified [18, 19]. These cells produce high concentrations of IL-10 which is considered as the main effector molecule of Breg-mediated immunosuppression [19, 20]. Bregs play an important role in autoimmune, transplantation, antitumour and other immune reactions. It has been shown that production of IL-10 by Bregs is regulated in a positive and negative manner by cytokines [21, 22]. Furthermore, we have shown that production of IL-10 by activated B cells is strongly inhibited by MSCs in the presence of IFN-γ [23]. Activation of cyclooxygenase-2 pathway and the synthesis of prostaglandin E_2 (PGE_2) has been identified as the mechanism of the suppression of IL-10 production by MSCs in the presence of IFN-γ [23]. We recently observed a similar inhibitory effect of MSCs on IL-10 production by B cells in the presence of IL-4. However, IFN-γ induces in MSCs the expression of a different spectrum of immunoregulatory molecules than IL-4 and the mechanisms of MSC-mediated suppression of IL-10 production are different in the presence of IL-4 or IFN-γ. It suggests that MSCs can inhibit not only the response to different antigens by distinct mechanisms, as it had been published [15], but also the response to the one stimulus by different mechanisms in dependence on the cytokine environment. These findings support the idea of the complexity of the immunoregulatory actions of MSCs.

Materials and Methods

Mice

Mice of both sexes of the inbred strain BALB/c were used in the experiments at the age of 7–9 weeks. The animals were purchased from the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague. The use of the animals was approved by the Local Ethical Committee of the Institute of Experimental Medicine.

MSC and B-Cell Enrichment Procedure

MSCs were prepared from bone marrow isolated from the femurs and tibias of female BALB/c mice. The bone marrow was flushed out and a single-cell suspension was seeded at a concentration of 4 x 10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) containing 10 % fetal calf serum (FCS, Gibco BRL, Grand Island, NY, USA), antibiotics (100 U/ml ml penicillin, 100 μg/ml ml streptomycin) and 10 mM Hepes buffer (hereafter referred to as complete DMEM) in 75-cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland). Nonadherent cells were washed out after 48 hrs of cultivation, and the remaining adherent cells were cultured for an additional 3 weeks (2–3 passages) at 37 °C in an atmosphere of 5 % CO_2. Plastic adherent cells were harvested by a short trypsinization and subsequent gentle scraping. The resulting cell suspension was incubated for 15 min with CD11b MicroBeads and CD45 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The cell suspension was then immunodepleted of $CD11b^+$ and $CD45^+$ contaminating cells using a magnetic activated cell sorter (AutoMACS, Miltenyi Biotec). The remaining $CD11b^-$ and $CD45^-$ cells were characterized in terms of their purity and differentiation potential.

For preparation of B cells, a single cell suspension of spleen cells was prepared in RPMI 1640 medium (Sigma Corp., St. Louis, CA, USA) containing 10 % FCS (Sigma), antibiotics (penicillin, streptomycin), 10 mM Hepes buffer and 5 x 10^{-5} M 2-mercaptoethanol. The B cells were isolated by a positive selection using the CD19 MicroBeads isolation kit (Miltenyi Biotec). The flow cytometry analysis showed that this procedure yielded a cell population containing more than 95 % $CD19^+$ cells and less than 2 % cells were $CD3^+$.

Characterization of Isolated B-Cell and MSC Populations

The purity and phenotype of the enriched B cell population were characterized by a flow cytometry using the following monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC)-labelled anti-CD19 (clone 6D5), Alexa Fluor 647-labelled anti-CD5 (clone 53–73), Alexa Fluor 647-labelled anti-CD22 (clone OX-97), phycoerythrin (PE)-labelled anti-CD1d (clone 1B1), allophycocyanine (APC)-labelled anti-CD11b (clone M1/70), and APC-labelled anti-CD3 (clone 17A2). All antibodies were purchased from BioLegend (San Diego, CA, USA).

To characterize the phenotype of MSCs, the cells were washed in PBS containing 0.5 % bovine serum albumin and then incubated for 30 min on ice with the following anti-mouse mAb: APC-labelled anti-CD44 (clone IM7, BD PharMingen, San Jose, CA, USA), PE-labelled anti-CD105 (clone MJ7/18, eBioscience, San Diego, CA, USA), PE-

labelled anti-CD73 (clone TY/11.8), eBioscience), APC-labelled anti-CD11b (clone M1/70, BioLegend) or FITC-labelled anti-CD45 (clone 30-F11, BioLegend). Dead cells were stained using Hoechst 33258 fluorescent dye (Invitrogen, Carlsbad, CA, USA) added to the samples 10 min before flow cytometry analysis. Data were collected using an LSRII cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). The morphological characteristics and differentiation potential of purified MSCs we have described in details elsewhere [23, 24].

Production and Detection of IL-10

B cells at a concentration 0.9 x 10^6 cells/ml were incubated in 48-well tissue culture plates (Corning Inc., Corning, NY, USA) in a final volume of 0.6 ml of complete RPMI 1640 medium unstimulated or stimulated with LPS (5 μg/ml, Difco Laboratories, Detroit, MI, USA). After a 72-hr incubation, the supernatants were harvested and tested for the presence of IL-10 by ELISA. To test effects of MSCs and IFN-γ on IL-10 production by B cells, MSCs at a ratio 1 : 30 or 10 ng/ml of recombinant mouse IL-4 or IFN-γ (PeproTech, Rocky Hill, NJ, USA) was added to the cultures of LPS-activated B cells and the production of IL-10 was determined.

The production of IL-10 was determined by ELISA using capture and detection mAb anti-IL-10 purchased from R & D Systems (Minneapolis, MN, USA) and following the instructions of manufacturer. The reaction was quantified by spectrophotometry using a Sunrise Remote ELISA Reader (Grōding, Austria).

Effects of Separation of B Cells and MSCs in a Transwell System on IL-10 Production

To test the effect of separation of MSCs from B cells on the suppression of IL-10 production, MSCs were separated from B cells by cell culture inserts (Nunc, Roskilde, Denmark) and cultured for 72 hrs in the presence of 5 μg/ml LPS and cytokines. The production of IL-10 in cultures of LPS-stimulated B cells alone, B cells mixed with MSCs or B cells separated from MSCs by semipermeabile membrane was determined.

In the other set of experiments, the supernatants were prepared by a 48-h incubation of untreated MSCs, or MSCs stimulated with 10 ng/ml of IL-4 or IFN-γ, LPS (5 μg/ml) or LPS plus IL-4 or IFN-γ. The supernatants were added to the cultures of B cells stimulated with LPS to make s final concentration 50 % of cell culture volume, and the production of IL-10 cells was determained after a 72-hr incubation.

Pretreatment of MSCs and B Cells with IL-4 or IFN-γ

MSCs (10^5 cells/well) were cultured in a volume 1 ml in 24-well tissue culture plates (Techno Plastic Products, Trasadinger, Switzerland) untreated or in the presence of 10 ng/ml of IL-4 or IFN-γ. After a 24-hr incubation, the adherent MSCs were washed with an excess of culture medium. B cells which were preincubated for 24 h alone or with 10 ng/ml of IL-4 or IFN-γ and then thoroughly washed, were added (0.9 x 10^6 cells/ml) to cultures of MSCs and were stimulated with LPS (5 μg/ml). The supernatants were harvested after a 72-h incubation and the concentrations of IL-10 were determined by ELISA.

Inhibition of MSC-Mediated Suppression of IL-10 Production

Purified B cells were stimulated with LPS in the presence of MSCs and IL-4 or IFN-γ and 1-methyl-D-tryptophane (1-MT, Sigma), i.e. selective inhibitor of indoleamine-2,3-dioxygenase (IDO), or inhibitor of Cox-2 synthesis indomethacine (Sigma), was added to the cultures to make a final concentration 10^{-3} M or 10^{-6} M, respectively. To block the progammed cell death-ligand 1 (PD-L1, CD274) – PD-1 (CD279) pathway, an inhibitory mAb anti-PD-L1 (clone MH5, eBioscience, San Jose, CA, USA) was added into the cultures at a concentration 5 μg/ml. To inhibit possible involvement of the Fas - FasL pathway in the immunosuppression, a mAb anti-Fas (anti-CD178, clone MFL3, eBioscience) was included into culture containing B cells, MSCs and cytokines at a final concentration 5 μg/ml.

Detection of Gene Expression

The expression of genes for IDO, Cox-2, trasnsforming growth factor-β (TGF-β), IL-6, hepatocyte growth factor (HGF), PD-L1, Fas and Fas-L was detected using real-time PCR, as we have described [23]. In brief, MSCs were cultured for 48 hrs unstimulated or stimulated with 10 ng/ml of IL-4 or IFN-γ, LPS (5 μg/ml) or LPS and IL-4 or IFN-γ, and total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. One μg of total RNA was treated with deoxyribonuclease I (Promega, Madison, WI, USA) and used for subsequent reverse transcription. The first-strand cDNA was synthesized using random hexamers (Promega) in a total reaction volume of 25 μl using M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) as we have previously described [23]. The sequences of primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), IDO, Cox-2, IL-6, TGF-β, HGF, PD-L1, Fas and FasL used for amplification are presented in Table 1. The PCR parameters included

Table 1 Murine primer sequences used for real-time PCR

Gene	Sense Primer	Antisense Primer
GAPDH	AGAACATCATCCCTGCATCC	ACATTGGGGGTAGGAACAC
IDO	GGGCTTTGCTCTACCACATC	AAGGACCCAGGGGCTGTAT
Cox-2	AGCCCACCCCAAACACAGT	AAATATGATCTGGATGTCAGCACATATT
IL-6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
TGF-β	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
HGF	CACCCCTTGGGAGTATTGTG	GGGACATCAGTCTCATTCACAG
PD-L1	CTACGGTGGTGCGGACTACA	CATGCTCAGAAGTGGCTGCAT
FasL	TGGGTAGACAGCAGTGCCAC	GCCCACAAGATGGACAGGG
Fas	GGCATCATTGGGCACTCCTT	GCTGCAAGCACAGCCTCTCT

denaturation at 95 °C for 3 min, 40 cycles at 95 °C for 20 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. Fluorescence data were collected at each cycle after an elongation step at 80 °C for 5 s and were analyzed using StepOne Software version 2.2.2 (Applied Biosystems).

Statistical Analysis

The results are expressed as the mean ± SD. Comparisons between two groups were analyzed by Student's *t*-test, and multiple comparisons were calculated by ANOVA. A value of $p < 0.05$ was considered statistically significant.

Results

Characterization of Purified B-Cell and MSC Populations

The MACS-purified B-cell and MSC populations were phenotypically characterized by flow cytometry. The purified B cell population contained more than 95 % $CD19^+$ cells and less than 2 % $CD3^+$ cells or $CD11b^+$ cells. Within $CD19^+$ cell populations, 93.8 ± 2.2 % cells were $CD19^+CD22^+$, 86.9 ± 1.8 % cells were $CD19^+CD1d^+$ and 7.6 ± 0.8 % cells were $CD19^+CD5^+$ (Fig. 1a). The growing MACS-purified MSCs had a typical fibroblast-like morphology, adhered to plastic and were positive with corresponding intensity for CD44, CD73 and CD105, which are markers attributed to murine MSCs (Fig. 1b). The MSC populations contained less than 1.0 % $CD11b^+$ cells and less than 3 % $CD45^+$ cells. In addition, MSCs were able to undergo adipogenic and osteogenic differentiation, when cultured in specific differentiation media, as we have described [24].

MSCs in the Presence of IL-4 or IFN-γ Strongly Inhibit IL-10 Production by B Cells

Purified B cells stimulated with LPS produced significant levels of IL-10 (Fig. 2). This production was even increased in the presence of IFN-γ and remained preserved or only slightly decreased in the presence of IL-4. Similarly, only a weak inhibitory effect on IL-10 production was detected, if B cells were stimulated with LPS in the presence of MSCs. However, a strong suppression of IL-10 production was observed when B cells were stimulated with LPS in the presence of MSCs and IL-4 (Fig. 2a) or MSCs and IFN-γ (Fig. 2b). The suppression was dependent on a concentration of MSCs (the suppression disappeared at ratios of MSCs cells to B cells below 1 : 30) and depended also on cytokine concentrations (data not shown).

The levels of IL-10 found in the supernatants after coculture of B cells and MSCs originate exclusively from stimulated B cells, since highly purified mouse MSCs (devoid of cells with leukocyte or myeloid cell markers) did not produce detectable IL-10 (data not shown).

The Suppression of IL-10 Production Requires Contact Between B Cells and MSCs

To test whether the MSC plus cytokine-mediated suppression of IL-10 production requires cell-cell contact or is caused by a cell-free molecule produced by MSCs, B cells were separated from MSCs by semipermeabile membrane and the production of IL-10 was determined. As demonstrated in Fig. 3a, separation of B cells and MSCs completely abrogated the suppression of IL-10 production occurring in the cultures containing MSCs and IL-4 or IFN-γ. In addition, the supernatants obtained after incubation of MSCs with IL-4, IFN-γ, LPS, LPS plus IL-4 or LPS plus IFN-γ did not suppress IL-10 production by LPS-stimulated B cells (Fig. 3b).

Expression of Genes for Immunoregulatory Molecules in MSCs Treated with IL-4 or IFN-γ

To search for the putative molecule(s) produced by MSCs and responsible for the suppression of IL-10 production by B cells, MSCs were cultured for 48 h alone or in the presence of IL-4, IFN-γ, LPS, LPS and IL-4 or LPS and IFN-γ, and the expression of genes for the putative immunomodulatory molecules was determined by RT-PCR. We identified three genes (*IDO, Cox-2* and *PD-L1*) which were upregulated in the presence of IFN-γ and one molecule (Fas) slightly upregulated in the

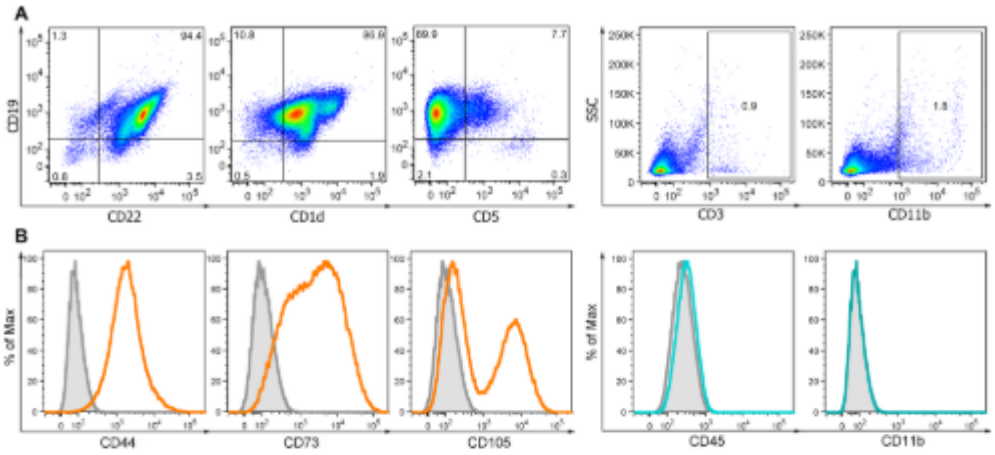

Fig. 1 The flow cytometry characterization of MACS-separated B-cell and MSC populations. Representative dot blots indicate the percentage of $CD19^+CD22^+$, $CD19^+CD1d^+$, $CD19^+CD5^+$, $CD3^+$ and $CD11b^+$ cells within the B-cell population (**a**). Histograms show the expression of CD44, CD73 , CD105, CD45 and CD11b markers within the MSCs (**b**). One of four similar experiments is shown

presence of IL-4 (Fig. 4). However, there were apparently distinct patterns of gene expression between IL-4- and IFN-γ – treated MSCs.

Distinct Effects of Preincubation with IL-4 or IFN-γ on the Immunosuppressive Properties of MSCs

Purified MSCs were preincubated for 24 h alone or in the presence of IL-4 or IFN-γ and then cultured with LPS-stimulated B cells. The suppression of IL-10 production occurred only in the cultures containing MSCs pretreated with IFN-γ (Fig. 5a). If B cells were preincubated alone or with IL-4 or IFN-γ and stimulated with LPS in the presence of MSCs, no significant suppression of IL-10 production was detected, irrespective of whether B cells were pretreated with IL-4 (Fig. 5b) or IFN-γ (Fig. 5c). Rather, B cells preincubated with IL-4 produced in the presence of MSCs even more IL-10 than in the absence of MSCs.

The Effects of Inhibitors on MSC-Mediated Suppression of IL-10 Production by B Cells

Since we identified 3 molecules (IDO, Cox-2 and PD-L1) which were upregulated in IFN-γ – treated MSCs and one

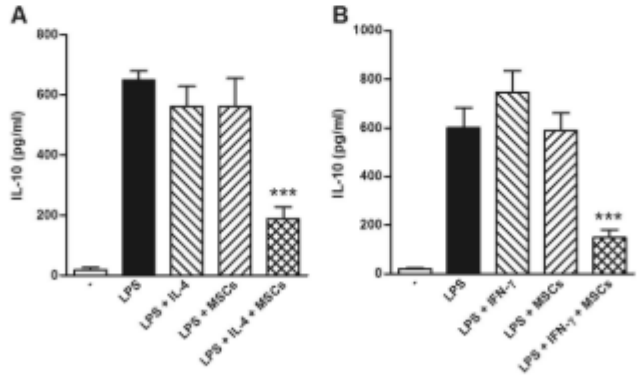

Fig. 2 The suppression of IL-10 production by LPS-stimulated B cells in the presence of MSCs and IL-4 (**a**) or MSCs and IFN-γ (**b**). Purified B cells were cultured unstimulated (−) or stimulated with LPS in the presence or absence of MSCs and/or IL-4 or IFN-γ. The production of IL-10 was determined in the supernatants after a 72-hr incubation period by ELISA. Each *bar* represents the mean ± SD from 4 independent experiments. Values with asterisks are significantly different ($^{***}p < 0.001$) from the control (B cells stimulated with LPS only)

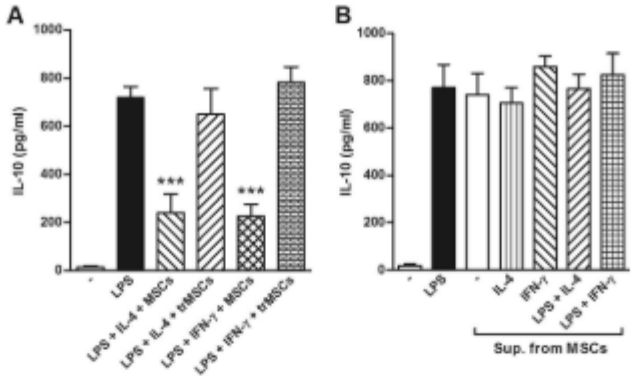

Fig. 3 The suppression of IL-10 production by B cells in cultures containing MSCs and IL-4 or IFN-γ requires contact between B cells and MSCs. (**a**) B cells were stimulated with LPS in the presence of MSCs and IL-4 or IFN-γ in standard culture conditions or in cultures where B cells were separated from MSCs (trMSCs) by a semipermeabile trasnswell system. (**b**) Production of IL-10 in cultures of B cells stimulated with LPS in the presence of supernatants obtained after cultivation of MSCs that were either unstimulated or stimulated for 48 h with IL-4, IFN-γ, LPS or LPS plus IL-4 or LPS plus IFN-γ. Each *bar* represents the mean ± SD from 3 independent experiments. Values with asterisks are significantly different (***$p < 0.001$) from the control (B cells stimulated with LPS only)

molecule (Fas) with slightly enhanced expression in IL-4-treated MSCs, we made attempts to block these molecules and thus inhibit their possible role in the suppression. We used indomethacine (an inhibitor of Cox-2 and PGE_2 synthesis), 1-MT (a selective inhibitor of IDO) or inhibitory mAb anti-PD-L1 and anti-Fas). These inhibitors were added to the cultures of B cells stimulated with LPS in the presence of MSCs and IL-4 or IFN-γ, and the production of IL-10 was determined by ELISA after a 72-h incubation period. The production of IL-10 was strongly inhibited in cultures containing MSCs and IL-4 or IFN-γ. As demonstrated in Fig. 6, indomethacine completely abrogated the suppression in cultures containing MSCs and IFN-γ, but had no detectable effect on the suppressioon in cultures containing MSCs and IL-4. The

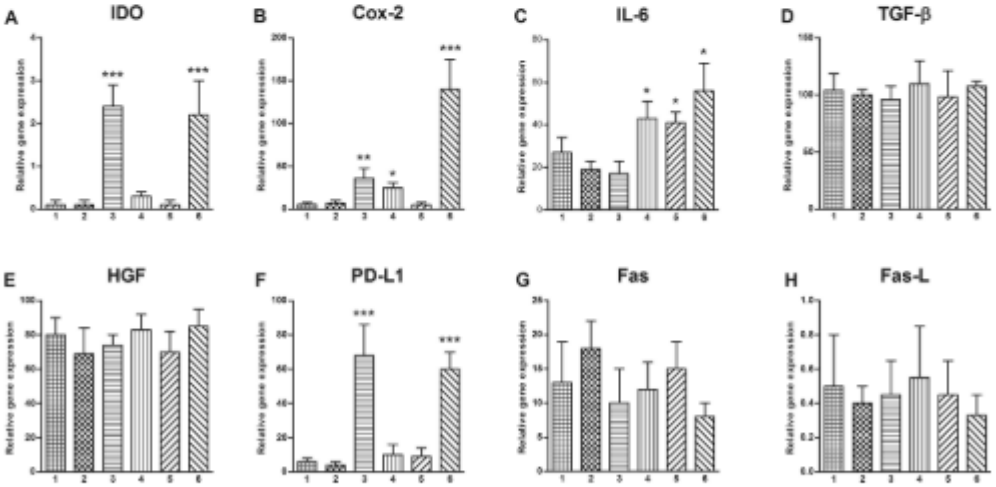

Fig. 4 The expression of genes for immunomodulatory molecules in purified MSCs which were cultured for 48 hrs unstimulated (*1*) or stimulated with IL-4 (*2*), IFN-γ (*3*), LPS (*4*) or with LPS and IL-4 (*5*) or LPS and IFN-γ (*6*). The expression of genes for IDO (**a**), Cox-2 (**b**), IL-6 (**c**), TGF-β (**d**), HGF (**e**), PDL-1 (**f**), Fas (**g**) or Fas-L (**h**) was determined by real-time PCR. Each *bar* represents the mean ± SD from 3 independent experiments. Values with asterisks are significantly different (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) from those for unstimulated MSCs

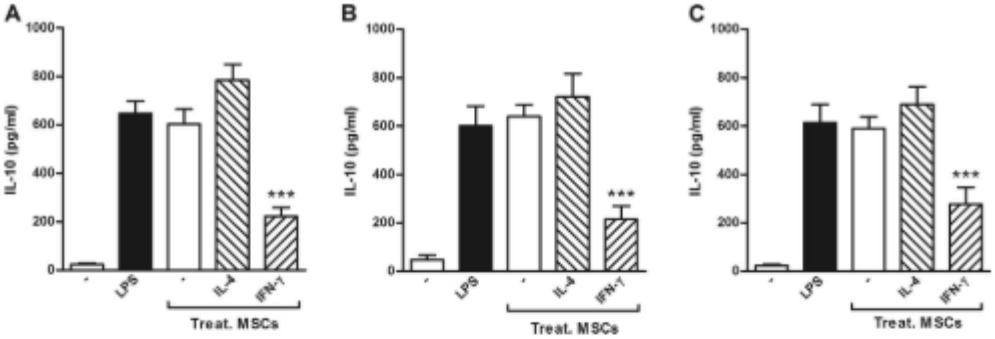

Fig. 5 The effects of MSCs which were preincubated with IL-4 or IFN-γ, on production of IL-10 by B cells. Purified MSCs were preincubated for 24 hrs untreated or in the presence of IL-4 or IFN-γ and were added to cultures of LPS-stimulated B cells that were either untreated (**a**) or preincubated with IL-4 (**b**) or IFN-γ (**c**). The production of IL-10 was determined after a 72-hr incubation by ELISA. Each *bar* represents the mean ± SD from 3 independent experiments. Values with *asterisks* are significantly different (***$p < 0.001$) from the control (B cells stimulated with LPS in the absence of MSCs)

suppression of IL-10 production in the cultures containing MSCs and IL-4 was not abrogated by any inhibitors or mAb.

Discussion

The immunomodulatory properties of MSCs have been well documented in various models and it has been shown that MSCs regulate the reactivity of T cells, macrophages, dendritic cells, NK cells and B cells by multiple different mechanisms. Although extensive data are available on the effects of MSCs on T cells, T cell subpopulations or antigen-presenting cells, less knowledge exists about the effects of MSC on B cells or even their subpopulations.

It has been shown that MSCs inhibit proliferation of B cells and decrease antibody production [25]. Within B-cell population, a subpopulation of cells producing IL-10 has been identified and called Bregs or B10 cells for their potential to inhibit immune responses by a production of IL-10 as a regulatory cytokine [20]. We recently described that the production of IL-10 by activated B cells is strongly suppressed by MSCs in the presence of IFN-γ [23]. This observation supported the findings that the immunomodulatory properties of MSCs depend on cytokine environment. It has been shown that MSCs sense their environment and can be polarized towards either a pro-

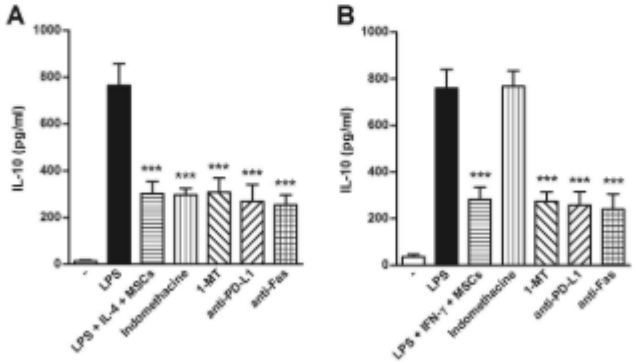

Fig. 6 The effects of IDO or Cox-2 inhibitors and neutralization mAb anti-PD-L1 or anti-Fas on the suppression of IL-10 production by B cells stimulated with LPS in the presence of MSCs and IL-4 or IFN-γ. Indomethacine (a selective inhibitor of Cox-2), 1-MT (an inhibitor of IDO), mAb anti-PD-L1 or mAb anti-Fas were added into the cultures of B cells stimulated with LPS in the presence of MSCs and IL-4 (**a**) or IFN-γ (**b**). The production of IL-10 was determined after a 72-hr incubation by ELISA. Each *bar* represents the mean ± SD from 3 independent experiments. Values with *asterisks* are significantly different (***$p < 0.001$) from the control (B cells stimulated with LPS in the absence of MSCs and IL-4 or IFN-γ)

inflammatory or anti-inflammatory phenotype depending on the TLR signals received [26, 27]. Other studies also confirmed the sensitivity of the immunoregulatory properties of MSCs to the cytokine environment [28–30]. Since the immunoregulatory activities of MSCs depend on their priming with cytokines, we tested the effects of other cytokines on MSC-mediated regulation of IL-10 production. The results revealed that IL-4 is another cytokine synergizing with MSCs in the suppression of IL-10 production by B cells, but that MSCs inhibit IL-10 production in the presence of IL-4 by a different mechanism than in the presence of IFN-γ.

The observation that MSCs, which are generally considered as immunosuppressive cells, inhibit production of immunosuppressive molecule IL-10, deserves more attention. Our data suggest that MSCs protect themselves (expression of inhibitory molecule PD-L1, production of suppressive molecules Cox-2 and IDO and in dependecnce on the cytokine environment modulate immune response to keep immunological homeostasis. MSCs suppress harmful immune responses, but simultaneously they can attenuate a strong immunosuppression by inhibition of IL-10 production. Multiple immunoregulatory mechanisms expressed in MSCs could ensure such complexity of immunomodulatory action of MSCs.

To search for a putative inhibitory molecule produced by IL-4- or IFN-γ − treated MSCs, different patterns of the expression of genes for immunoregulatory molecules were found. While IFN-γ stimulated the expression of *IDO*, *Cox-2* and *PD-L1* genes, IL-4 had no effect on the expression of these genes, but rather enhanced the expression of Fas molecule. While the involvement of IDO or Cox-2 in MSC-mediated immunosuppression has been well documented [8, 12], the high expression of PD-L1 molecules on IFN-γ-treated MSC might suggest the role of PD-L1 in the protection of MSCs and in their lower immunogenicity. Similarly, the expression of PD-L1 was found in some types of cancers, where this molecule contributes to their low immunogenicity and has become a target for cancer immune therapy [31, 32]. The use of selective inhibitors or mAb against PD-L1 or Fas molecules showed that only indomethacine, a selective inhibitor of Cox-2 and PGE_2 production, completely abrogated IFN-γ-induced suppression. However, indomethacine had no effect on the IL-4-induced suppression of IL-10 production. The results also showed that MSC-mediated suppression of IL-10 production in the presence of IFN-γ or IL-4 was completely abrogated, if B cells and MSCs were separated by semipermeabile membrane. Furthermore, MSCs preincubated with IFN-γ and then carefully washed strongly inhibited IL-10 production by B cells. On the other hand, MSCs preincubated with IL-4 rather enhance IL-10 secretion, but the incubation of B cells with MSCs and IL-4 simultaneously strongly inhibited IL-10 production. These observations suggest that IL-4 and IFN-γ activate different regulatory mechanisms in MSCs, one of which involves the Cox-2 - PGE_2 pathway and other remains to be resolved. Even Akiyama et al. [33] described the involvement of Fas/FasL-induced apoptosis in MSC-mediated immunosuppression and we observed the enhanced Fas expression in IL-4-treated MSCs, we were not able to abrogate the suppression of IL-10 production by inclusion of anti-Fas antibodies into cultures containing B cells, MSCs and IL-4.

It has been well documented that MSCs modulate immune response by multiple mechanisms. Some of these mechanisms (TGF-β, HGF or IL-6 production, Fas and FasL expression)

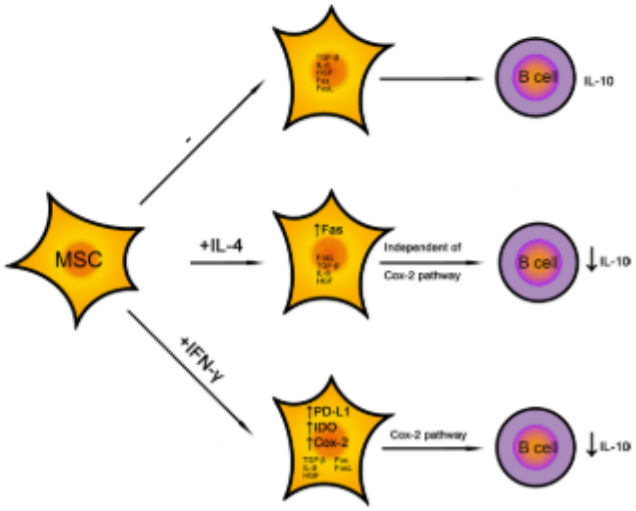

Fig. 7 Immunoregulatory mechanisms activated in MSCs by IL-4 or IFN-γ. Untreated MSCs spontaneously express genes for TGF-β, IL-6, HGF, Fas and FasL, but do not inhibit IL-10 production by LPS-stimulated B cells. In the presence of IL-4, an upregulation of *Fas* gene can be detected and MSCs inhibit IL-10 production. In the presence of IFN-γ MSCs strongly upregulate genes for IDO, Cox-2 and PD-L1 molecules and inhibit IL-10 production. The inhibition of IL-10 production by IFN-γ-activated MSCs involves the Cox-2 pathway, while the suppression of IL-10 production by IL-4-activated MSCs is Cox-2 independent

are constitutively expressed by MSCs, while others (such IDO, PD-L1, Cox-2) are activated only after stimulation with cytokines or through Toll-like receptors [12]. We have shown that two of these mechanisms are involved in the MSC-mediated suppression of IL-10 production by activated B cells. As it is summarized in Fig. 7, one inhibitory mechanism is induced by the proinflammatory cytokine IFN-γ and involves the Cox-2 pathway. The other mechanism is activated by the Th2 cytokine IL-4 and is independent of Cox-2. These observations show that MSCs can inhibit response to the same stimulus by different mechanisms in dependence on cytokine environment. The findings thus support idea of the complexity of the mechanisms of MSC-mediated immunosuppression.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest

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4.3. IL-10 production by B cells is regulated by cytokines, but independently of GATA-3 or FoxP3 expression

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Besides the production of antibodies, B cells can also regulate the immune response in an antibody-independent manner. The hallmark mechanism of action of Bregs seems to be the production of IL-10, but knowledge about the regulation of IL-10 production by Bregs remains limited. In this study, we described that purified mouse LPS-stimulated B cells produce significant levels of IL-10, and this production is regulated by cytokines.

In LPS-stimulated B cells, IL-10 production was significantly enhanced by IFN-γ and decreased by IL-4 or TGF-β, lineage-specific cytokines that play a role in the generation of Th2 and Treg cells. These findings are in sharp contrast with observations in concanavalin A (ConA)-stimulated T cells, where these cytokines regulate IL-10 production in a reverse manner than in LPS-stimulated B cells. Moreover, in T cells, the production of IL-10 is regulated by GATA-3 and FoxP3 transcription factors. We investigated the possibility of a role of these factors in IL-10 production in B cells because $FoxP3^+$ Bregs were described. The results showed that B cells did not express T cell lineage-specific transcription factors, although the expression of these factors was associated with IL-10 production by T cells. However, the expression of IL-10 correlated with the expression of transcription factor HIF-1α in LPS-stimulated B cells under IFN-γ conditions. ConA-stimulated T cells also expressed the transcription factor HIF-1α, but the tested cytokines do not influence its expression in T cells.

These observations showed that antagonistic cytokines IFN-γ, IL-4, and TGF-β for lineage-specific T cell development modified IL-10 expression in B cells in a reverse manner than in T cells. Additionally, the production of IL-10 by Bregs was independent of transcription factors GATA-3 and FoxP3, which regulate IL-10 production by $GATA\text{-}3^+$Th2 cells and $FoxP3^+$ Tregs.

Aspirant's contribution: sample analyses, data analyses and interpretation, manuscript writing.

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Interleukin-10 production by B cells is regulated by cytokines, but independently of GATA-3 or FoxP3 expression

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ABSTRACT

The knowledge of mechanisms of regulation of IL-10 production by B cells remains still very limited. We show here that highly purified mouse B cells stimulated with LPS produce significant levels of IL-10, but Bregs in our model do not express detectable level of either Foxp3 or GATA-3. Nevertheless, IL-10 production by B cells is regulated by cytokines. In activated B cells, IL-10 production was significantly enhanced by IFN-γ and decreased in the presence of IL-4 or TGF-β. These findings are in sharp contrast with the observations in T cells, where IL-10 production correlates with GATA-3 or FoxP3 expression, and the cytokines regulate IL-10 production in a reverse manner than in activated B cells. These results thus show that the production of IL-10 by Bregs is regulated by cytokines independently of the expression of GATA-3 and FoxP3, which is clearly different from GATA-3-dependent IL-10 production by activated Th2 cells and FoxP3 expression in IL-10-producing Tregs.

1. Introduction

The development and reactivity of individual B- and T-cell subpopulations depend on the expression of lineage-specific transcription factors, which characterize a particular cell type. Although extensive knowledge exists about the differentiation and function of T-cell subpopulations, much less is known about the role of cytokines and transcription factors in the development and function of individual B-cell types. Within the B-cell population, a subset of cells producing relatively high concentrations of IL-10, has been identified. These cells inhibit autoimmune, inflammatory and transplantation reactions, and for these functions they are called Bregs [1]. So far, limited knowledge exists about the activation of Bregs and about the regulation of IL-10 production by B cells. It has been demonstrated that production of IL-10 by B cells can be induced by stimulation through Toll-like receptors, B-cell receptor or B-cell mitogens [2–5], and that this production is regulated in a positive and negative manner by cytokines [3,6,7]. We have shown that IL-10 production by activated B cells is enhanced by IL-12 or IFN-γ and decreased in the presence of IL-21 or TGF-β [3].

Within the T-cell compartment, Th2 and Treg subpopulations produce substantial levels of IL-10. The development of Th2 cells and the production of IL-10 are also strictly regulated by cytokines. Originally, IL-4 was shown to be the most important factor determining the development of Th2 cells and supporting the secretion of IL-10 [8]. On the molecular level, transcription factor GATA-3 has been identified as the master regulator of Th2-cell differentiation, and also plays a crucial role in the activation of IL-10 production by Th2 cells [9]. The transcription factor FoxP3 has been identified as an important regulator for the development of Tregs [10]. FoxP3 is constitutively expressed in natural $CD4^+CD25^+$ Tregs and in activated Tr-1 regulatory T cells which produce a high amount of IL-10. Furthermore, signals provided by TGF-β are essential for the development of Tregs and for their homeostasis and regulatory functions [11,12]. It has been suggested that B cells can also express the transcription factor FoxP3 and that $FoxP3^+$ B cells could represent a subpopulation of Bregs [13]. However, the expression of FoxP3 and its role in B cells remains unclear and controversial [14]. It has been also shown that hypoxia-inducible factor-1α (HIF-1α) could play role in IL-10 expression in B cells [15].

Based on our experience in the study of IL-10 production by Bregs [3,16], we investigated the role of cytokines and the transcription

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factors GATA-3 and FoxP3 in IL-10 production by LPS-stimulated B cells. We found that IL-10 production by B cells is regulated by cytokines, but is independent of GATA-3 or FoxP3 expression. In contrast to IL-10 production by B cells, Con A-stimulated T cells expressed GATA-3 or FoxP3 in dependence on the cytokine stimulation, and IL-10 production in T cells is correlated with the expression of GATA-3 or FoxP3.

2. Materials and methods

2.1. Mice

Female BALB/c mice at the age of 10–12 weeks were used in the experiments. The animals were purchased from the breeding unit of the Institute of Molecular Genetics of the Czech Academy of Sciences, Prague. The use of the animals was approved by the Local Ethical Committee of the Institute of Experimental Medicine.

2.2. B-cell and T-cell enrichment procedures

Single cell suspensions of spleen cells were prepared in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (Gibco BRL, Grand Island, NY), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, Sigma-Aldrich), 10 mM HEPES buffer (Sigma-Aldrich) and 5×10^{-5} M 2-mercaptoethanol (Serva, Heidelberg, Germany), hereinafter a complete RPMI-1640 medium. To isolate the B-cell population, the spleen cell suspension was incubated for 15 min with CD19 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. B cells were isolated by a positive selection using a magnetic activated cell sorter (AutoMACS, Miltenyi Biotec). The purity and phenotype of cells were characterized by flow cytometry.

For the preparation of T cells, a single cell suspension of spleen cells was prepared in complete RPMI-1640 medium, and the population of T cells was isolated by a negative selection using a Pan T Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer's instructions. Flow cytometry was used for characterization of the final cell population.

2.3. Characterization of isolated B-cell and T-cell populations

To characterize the purity and phenotype of the enriched B-cell population, the cells were incubated for 30 min at 4 °C with the following monoclonal antibodies (mAb): fluorescein isothiocyanate (FITC)-conjugated anti-CD19 (clone 6D5), Alexa Fluor 647-conjugated anti-CD22 (clone OX-97), PerCP-Cy5.5-conjugated anti-B220 (CD45R) (clone RA3-6B2), allophycocyanin (APC)-conjugated anti-CD3 (clone 17A2) and phycoerythrin (PE)-conjugated anti-CD14 (clone Sa14-2), and analyzed by flow cytometry. All antibodies were purchased from Biolegend (San Diego, CA).

The purity and phenotype of the enriched T-cell population was characterized using the following mAb: APC-conjugated anti-CD3 (clone 17A2), FITC-conjugated anti-CD4 (clone GK1.5), PE-conjugated anti-CD8 (clone 53-6.7), FITC-conjugated anti-CD19 (clone 6D5) and PE-conjugated anti-CD14 (clone Sa14-2). Before flow cytometry analysis, dead cells were stained by adding Hoechst 33258 fluorescent dye (Invitrogen, Carlsbad, CA) to the samples 15 min before flow cytometry analysis. Data were collected by LSR II cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo software (LLC, Ashland, OR).

2.4. Production and detection of IL-10

Purified B cells at a concentration of 0.6×10^6 cells/ml, or T cells at a concentration of 0.5×10^6 cells/ml with added peritoneal exudate macrophages (as a source of antigen-presenting cells, a ratio of T cells to macrophages 10:1), were incubated in 48-well tissue plates (Corning Inc., Corning, NY) in a final volume of 1 ml of complete RPMI-1640 medium, unstimulated or stimulated with B-cell mitogen LPS from *Escherichia coli* 0127:B8 (10 μg/ml, Sigma-Aldrich) or T-cell mitogen Con A (1 μg/ml, Sigma-Aldrich). To test the effects of cytokines on IL-10 production, mouse recombinant IFN-γ, IL-4 or TGF-β (PeproTech, Rocky Hill, NJ) was added to the cultures in a final concentration of 10 ng/ml for IFN-γ and IL-4 and 1 ng/ml for TGF-β. After a 48-h incubation period for B cells and a 72-h incubation for T cells at 37 °C in an atmosphere of 5% CO_2, the supernatants were harvested and tested for the presence of IL-10.

The production of IL-10 was determined by ELISA using capture and detection anti-IL-10 mAb, purchased from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. The reaction was quantified by Sunrise Remote ELISA Reader (Gröding, Austria).

2.5. Intracellular detection of IL-10, GATA-3 and FoxP3

The intracellular expression of IL-10, GATA-3 and FoxP3 was analyzed by flow cytometry. The isolated B-cell or T-cell populations were stimulated with LPS (10 μg/ml) or Con A (1 μg/ml) in the absence or presence of IFN-γ (10 ng/ml), IL-4 (10 ng/ml) or TGF-β (1 ng/ml). Phorbol myristate acetate (20 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml, Sigma-Aldrich) and Brefeldin A (5 μg/ml, eBioscience, San Diego, CA) were added to the cultures for the last 4 h of the 48-h incubation period. The dead cells were stained with a Zombie Violet Fixable Viability Kit (1:500; BioLegend) before intracellular staining. Cells were stained with PE-conjugated anti-CD19 mAb (clone 6D5, BioLegend) or PE-conjugated anti-CD3 mAb (clone 17A2, BioLegend) and then permeabilized using a FoxP3 Staining Buffer Set (eBioscience), according to the manufacturer's instructions. The cells were stained intracellularly with APC-conjugated anti-IL-10 mAb (clone JES5-16E3; eBioscience), Alexa Fluor 488-conjugated anti-GATA-3 mAb (clone TWAJ, eBioscience) or Alexa Fluor 488-conjugated anti-FoxP3 mAb (clone 150D/E4, eBioscience) and analyzed with a LSRII flow cytometer (BD Bioscience). All flow cytometry experiments were gated on viable, single lymphocytes and data were analyzed using FlowJo software (Treestar, Ashland, OR).

2.6. Detection of gene expression

The expression of genes for IL-10, GATA-3 and FoxP3 was detected using real-time PCR. B cells at a concentration of 0.6×10^6 cells/ml and T cells at a concentration of 0.5×10^6 cells/ml (with peritoneal exudates macrophages at a ratio of 10:1) were incubated for 48 h unstimulated or stimulated with LPS (10 μg/ml) or Con A (1 μg/ml), and with or without IFN-γ (10 ng/ml), IL-4 (10 ng/ml) or TGF-β (1 ng/ml). The total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH). One microgram of RNA was treated with deoxyribonuclease I (Promega, Madison, WI) and used for the subsequent reverse transcription. The first-strand cDNA was synthesized using random hexamers (Promega) by M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed in a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA), as we have previously described [17] and was analyzed using StepOne Software version 2.2.3 (Applied Biosystems). The following primers were used for amplification: *Il-10* gene (sense: ATTTGAATTCCCTGGGTGAGAAG, antisense: CACAGGGAGAAATCGATGACA), *Gata-3* (sense: TTATCAAG CCCAAGCGAAG, antisense: TGGTGGTGGTCTGACAGTTC), *FoxP3* gene (sense: GGCCCTTCTCCAGGACAGA, antisence: GCTGATCATGGCTGG GTTGT), *Hif1a* gene (sense: GCTTACACACAGAAATGGCC, antisense: AGCACCTTCCACGTTGCTGA), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene (sense: AGAACATCATCCCTGCATCC, antisense: ACATTGGGGGTAGGAACAC) and *IL-10 receptor α (Il-10Rα) (CD210a)* gene (sense: CGCTTGGAATCCCGAATTA, antisense: CTCAG GTTGGTCACAGTGAAAT). A relative quantification model was applied to calculate the expression of the target gene in comparison to *Gapdh*, used as an endogenous control.

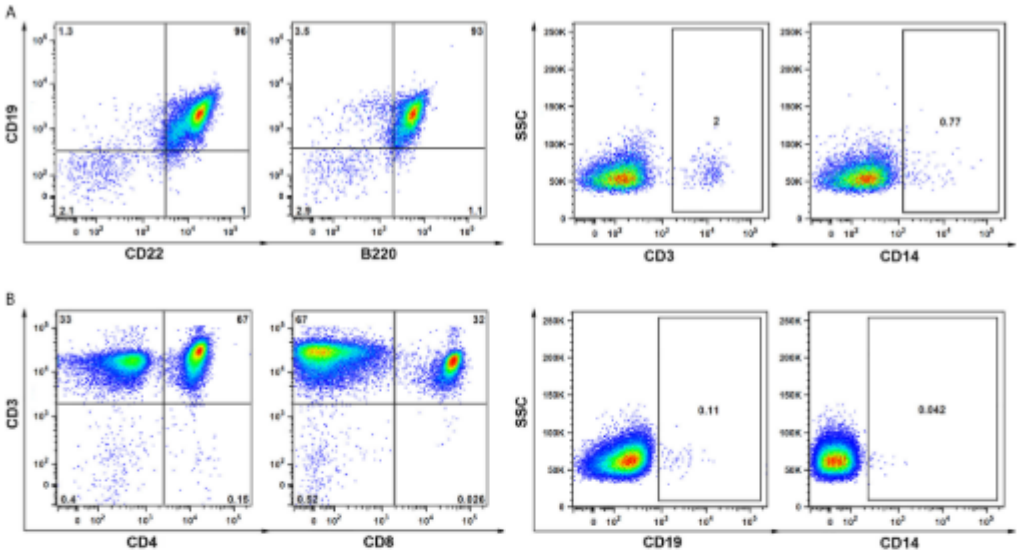

Fig. 1. Phenotypic characterization of enriched B-cell (A) and T-cell (B) populations. The cells were separated using a magnetic activated cell sorter and their phenotype was characterized by flow cytometry. Representative dot plots indicate the percentage of $CD19^+CD22^+$, $CD19^+B220^+$, $CD3^+$ and $CD14^+$ cells among the B-cell population, and the percentage of $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$ and $CD14^+$ cells among the T-cell population. One typical experiment of three similar ones is shown.

2.7. Statistical analysis

The results are expressed as the mean ± SD. Comparisons between the two groups were analyzed by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Phenotypic characterization of purified B-cell and T-cell populations

Purified spleen B-cell and T-cell populations were phenotypically characterized by flow cytometry. As demonstrated in Fig. 1, the isolated B-cell population contained more than 96% $CD19^+$ cells. This population included approximately 96% $CD19^+CD22^+$ and 93% $CD19^+B220^+$ cells, and < 2% cells were $CD3^+$ and about 1% cells were $CD14^+$ (Fig. 1A). The enriched T-cell population contained about 99% $CD3^+$ cells which included approximately 67% $CD3^+CD4^+$ and 32% $CD3^+CD8^+$ cells, and < 0.5% of cells were $CD19^+$ or $CD14^+$ (Fig. 1B).

3.2. The different roles of GATA-3 and FoxP3 in IL-10 production by B and T cells

As has been previously described, GATA-3 represents the key transcription factor for IL-10 production in Th2 cells, and FoxP3 has been identified as a crucial transcription factor for Tregs and their regulatory functions [10,18]. To explore the role of GATA-3 and FoxP3 in the regulation of IL-10 production by activated B cells, the cells were cultured for 48 h unstimulated or stimulated with LPS in the absence or presence of IFN-γ, IL-4 or TGF-β, and the expression of IL-10 and GATA-3 or FoxP3 proteins was determined by intracellular staining using flow cytometry. Fig. 2 shows representative dot plots from the studies of co-expression of IL-10 and GATA-3 or FoxP3 in activated B and T cells. LPS induced the expression of IL-10 in B cells, and this expression was modulated by cytokines. In this respect, IFN-γ enhanced the percentage of $IL\text{-}10^+$ B cells while IL-4 or TGF-β rather decreased the proportion of $IL\text{-}10^+$ B cells. The production of IL-10 was independent of GATA-3 or FoxP3 expression and activated B cells did not express detectable levels of GATA-3 or FoxP3 (Fig. 2A, B). On the contrary, the production of IL-10 by Con A-stimulated T cells correlated with the expression of GATA-3 or FoxP3 (Fig. 2C, D). In the presence of IL-4, the expression of IL-10 correlated with the expression of GATA-3, as demonstrated by a total of 9.5% $GATA\text{-}3^+IL\text{-}10^+$ T cells. Similarly, TGF-β enhanced the percentage of $FoxP3^{hi}IL\text{-}10^+$ T cells. IFN-γ did not influence IL-10 expression in activated T cells. The quantitative demonstration and statistical analysis of these observations are summarized in Fig. 3. The figure shows that the expression of IL-10 in B cells was independent (< 0.05% $IL\text{-}10^+GATA\text{-}3^+$ or $IL\text{-}10^+FoxP3^+$ B cells) of the expression of GATA-3 or FoxP3 (Fig. 3B, E). The proportion of $IL\text{-}10^+GATA\text{-}3^-$ or $IL\text{-}10^+FoxP3^-$ activated B cells was significantly increased in the presence of IFN-γ, while IL-4 and TGF-β had the opposite effect on the percentage of these cells (Fig. 3A, D). In the T-cell compartment, only a small proportion (< 1%) of Con A-stimulated T cells expressed IL-10 in the absence of cytokines (Fig. 3G, J). TGF-β significantly increased the percentage of $IL\text{-}10^+FoxP3^{hi}$ T cells (Fig. 3H). Nevertheless, most of highly positive $FoxP3^{hi}$ T cells were not simultaneously $IL\text{-}10^+$ (Fig. 3I). IL-4 significantly enhanced the proportion of $IL\text{-}10^+GATA\text{-}3^+$ T cells up to 10% (Fig. 3K), but a high percentage of $GATA\text{-}3^+$ T cells did not express IL-10 (Fig. 3L). Altogether, the results show that IL-10 production by B cells is not associated with the expression of GATA-3 or FoxP3.

3.3. The effects of IFN-γ, IL-4 and TGF-β on Il-10, Gata-3, FoxP3 and Hif1a gene expression in mitogen-stimulated B and T cells

To test whether the effects of IFN-γ, IL-4 and TGF-β on the expression of IL-10, FoxP3 and GATA-3 occurred already at the level of Il-10, Gata-3 and FoxP3 gene expression, purified B or T cells were

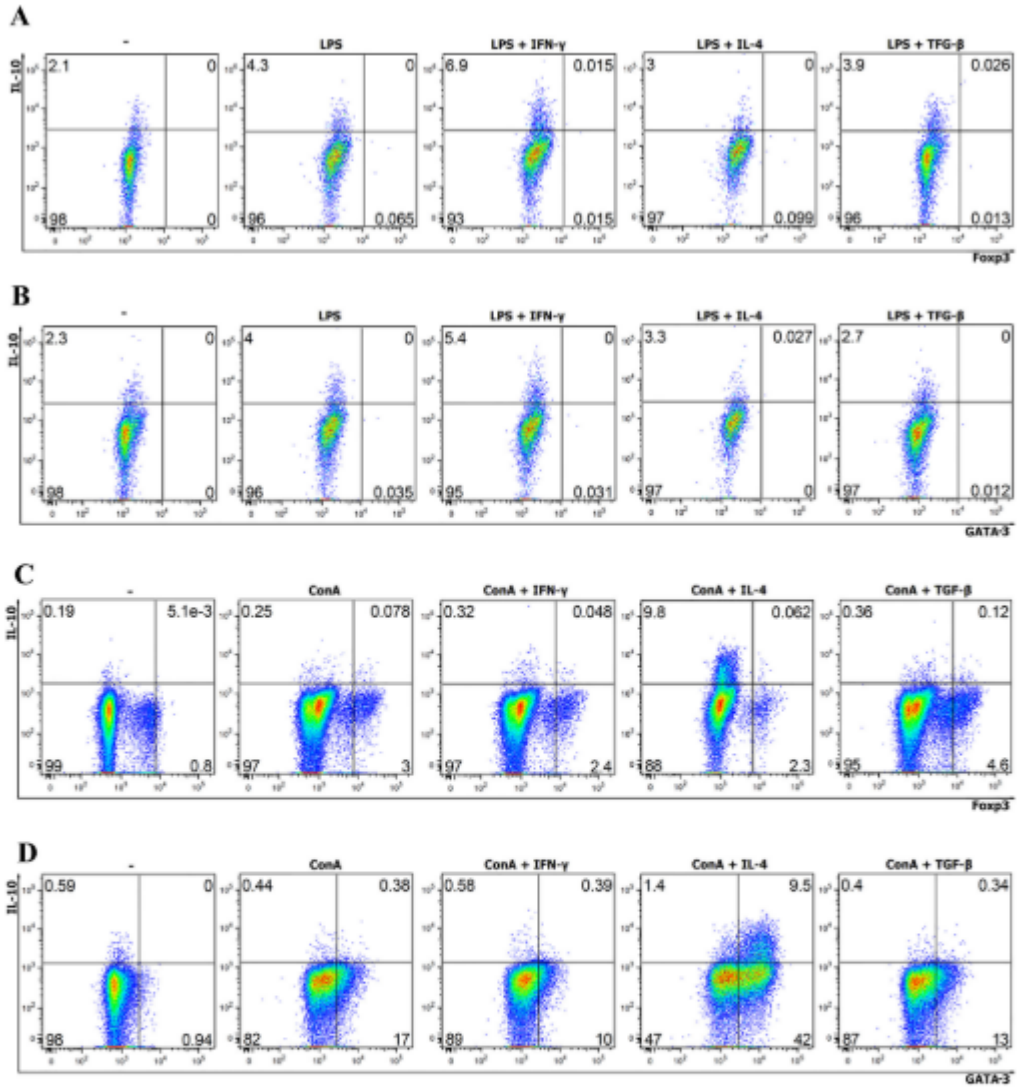

Fig. 2. Representative dot plots demonstrating the percentage of IL-10$^+$, GATA-3$^+$ and FoxP3^{hi} B and T cells. Purified CD19$^+$ B cells (A, B) and enriched CD3$^+$ T cells (C, D) were cultured unstimulated (−) or stimulated with mitogens in the absence or presence of IFN-γ, IL-4 or TGF-β. The percentage of IL-10$^+$, GATA-3$^+$ and FoxP3^{hi} CD19$^+$ or CD3$^+$ cells was determined by intracellular staining using flow cytometry. One representative dot plot from four independent experiments is shown.

stimulated for 48 h with mitogens in the absence or presence of cytokines and the expression of genes was determined by real-time PCR. As demonstrated in Fig. 4A, IFN-γ enhanced expression of the gene for IL-10 in B cells. On the other hand, in the presence of IL-4 or TGF-β, the level of mRNA for IL-10 was rather decreased (Fig. 4A). The expression of *Gata-3* and *FoxP3* genes in LPS-stimulated B cells was almost not detected (Fig. 4B, C). On the contrary, IL-4 strongly enhanced both *Il-10* and *Gata-3* gene expression in Con A-stimulated T cells (Fig. 4E, G). Similarly, TGF-β also significantly increased the gene expression for IL-10 and strongly enhanced *FoxP3* gene expression (Fig. 4E, F). IFN-γ had no effect on the *Il-10, Gata-3* and *FoxP3* gene expression in activated T cells (Fig. 4E-G). These results demonstrated that IFN-γ, IL-4 and TGF-β regulate differently the expression of genes for IL-10, GATA-3 and FoxP3 in mitogen-stimulated B and T cells.

Since association between production of IL-10 and the expression of HIF-1α has been described [15], we tested the expression of HIF-1α in

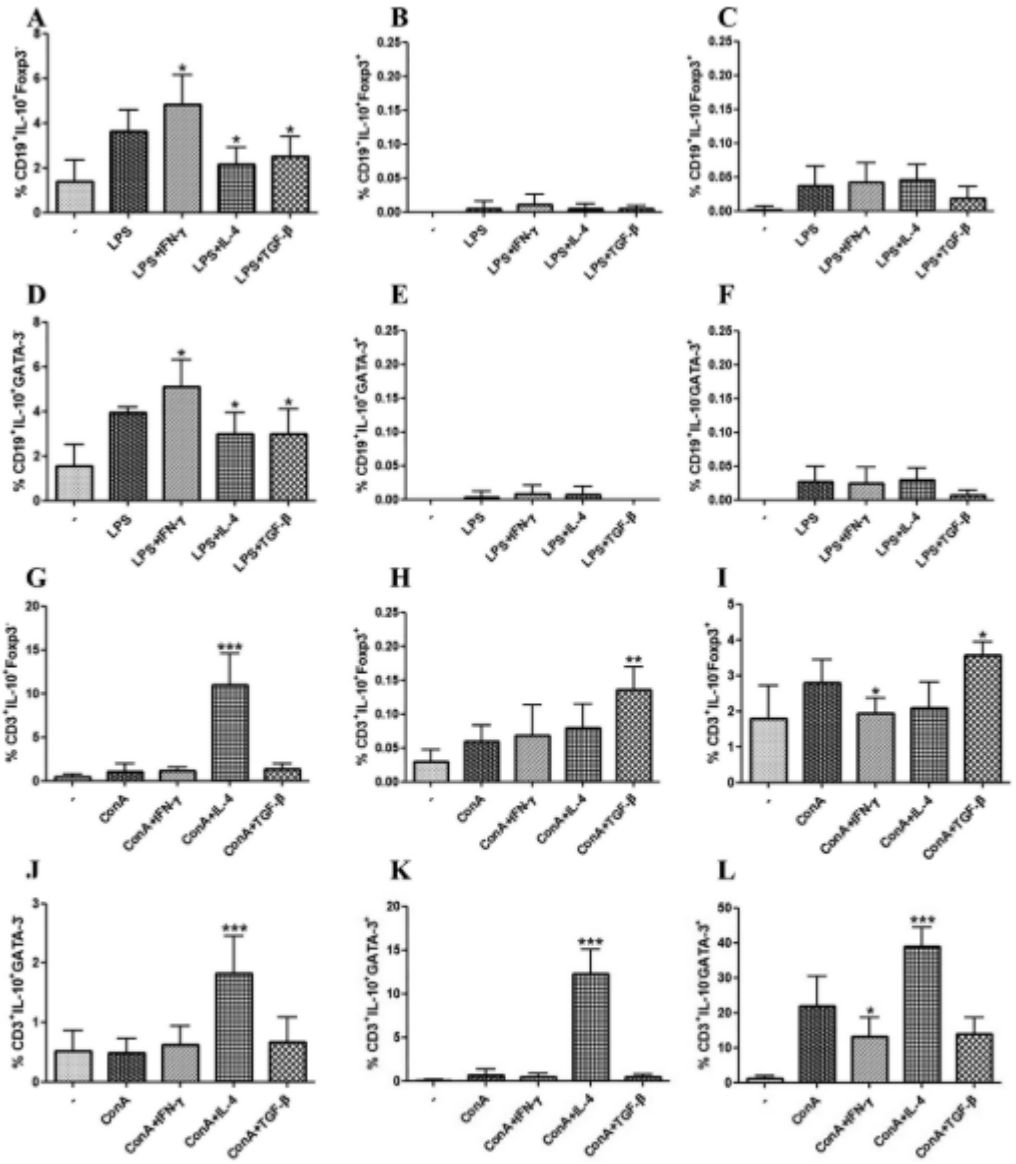

Fig. 3. The effect of cytokines on the percentage of IL-10$^+$, GATA-3$^+$, IL-10$^+$GATA-3$^+$, FoxP3^{hi} and IL-10$^+$FoxP3^{hi} B or T cells. Purified B (A–F) were cultured for 48 h and T (G–L) cells were cultured for 72 h unstimulated (−) or stimulated with mitogens in the absence or presence of IFN-γ, IL-4 or TGF-β. The percentage of IL-10$^+$ and GATA-3$^+$ or FoxP3$^+$ cells was determined by intracellular staining using flow cytometry. Each bar represents the mean + SD from four independent determinations. Values with asterisks are significantly different ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) from the control (the cells stimulated with mitogen in the absence of added cytokines).

mitogen-stimulated B and T cells by real-time PCR. As demonstrated in Fig. 4D, IFN-γ enhanced expression of the gene for HIF-1α in LPS-stimulated B cells, but IL-4 and TGF-β did not modify HIF-1α expression in B cells. On the contrary, neither IFN-γ, IL-4 nor TGF-β had effect on HIF-1α expression in Con A-stimulated T cells (Fig. 4H).

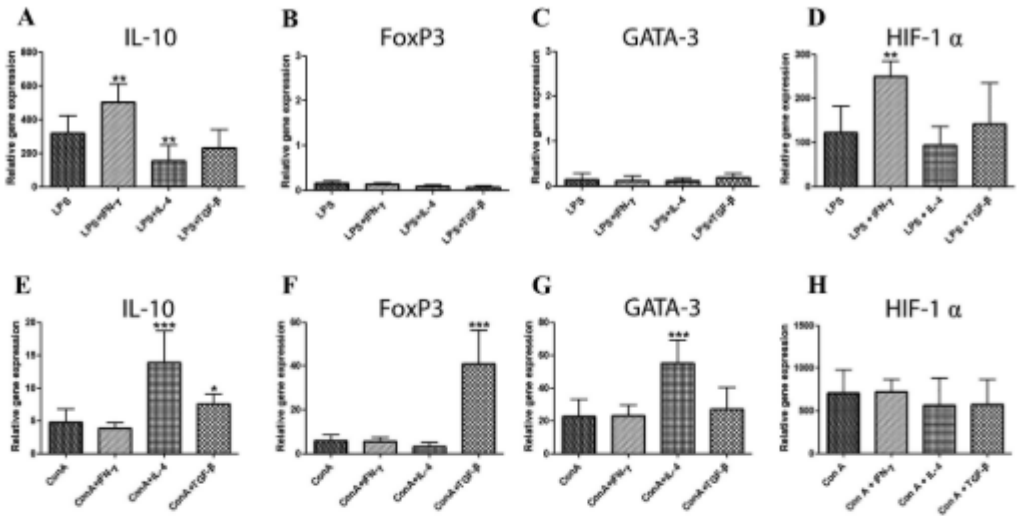

Fig. 4. Expression of genes for IL-10, GATA-3, FoxP3 and HIF-1α in mitogen-stimulated B and T cells. Purified B (A–D) or T (E–H) cells were stimulated for 48 h with LPS or Con A in the absence or presence of IFN-γ, IL-4 or TGF-β. The expression of genes for IL-10, GATA-3, FoxP3 or HIF-1α was determined by real-time PCR. Each bar represents the mean + SD from three independent determinations. Values with asterisks are significantly different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) from the control (the cells stimulated with mitogen in the absence of added cytokines).

3.4. The effects of IFN-γ, IL-4 and TGF-β on IL-10 production by mitogen-stimulated B and T cells

To test the effects of IFN-γ, IL-4 and TGF-β on IL-10 production by B and T cells, the cells were cultured for 72 h unstimulated or stimulated with LPS or Con A in the absence or presence of the cytokines, and the concentrations of IL-10 in the supernatants were detected by ELISA. LPS-stimulated B cells produced a substantial level of IL-10 and this production was significantly enhanced in the presence of IFN-γ, while IL-4 and TGF-β decreased IL-10 production (Fig. 5A). On the other hand, IL-10 production by Con A-stimulated T cells was significantly enhanced in the presence of IL-4, but the level of secreted IL-10 in the presence of TGF-β was not enhanced (Fig. 5B).

There was a discrepancy between the level of secreted IL-10 as determined by ELISA (no effect of TGF-β on IL-10 production in T cells) and by the intracellular expression of IL-10 as determined by flow cytometry (a significant increase in the proportion of IL-10^+ T cells). To test the possibility that this difference could be due to absorption out of secreted IL-10 by IL-10R on purified T cells, the cells were stimulated for 48 h in the absence or presence of cytokines, and *Il-10ra* gene expression was determined by real-time PCR. As demonstrated in Fig. 5C, TGF-β (but not IL-4) significantly enhanced the expression of the gene for IL-10Ra. This result indicates that the difference between the secretion of IL-10 protein and the intracellular staining of IL-10^+ cells could be due to a reverse uptake of secreted IL-10 by the enhanced expression of IL-10R on T cells activated in the presence of TGF-β.

4. Discussion

The antibody-independent immunoregulatory function of B cells is mediated mainly by the production of suppressive cytokine IL-10 [1,4,5]. It has been shown that IL-10 is produced by a population of Bregs which inhibits autoimmune, inflammatory, anti-cancer and transplantation reactions [19]. The production of IL-10 by B cells is regulated in both a positive and negative manner by cytokines [3,7]. In the present study, we selected IFN-γ, IL-4 and TGF-β as cytokines which have different effects on the development of Th1, Th2 and Treg cells and on the polarization of immunological reactivity [20–22]. We have shown here that these cytokines also have different effects on IL-10 production by B and T cells. IFN-γ, which supports pro-inflammatory Th1 responses, increased *Il-10* gene expression, enhanced the percentage of IL-10-producing B cells and increased the level of secreted IL-10 by B cells. By contrast, IL-4 and TGF-β which determine the development of IL-10-producing Th2 and Treg cells, rather decreased the production of IL-10 by activated B cells. The different regulation of B and T cells by cytokines might represent an important negative feedback loop in which pro-inflammatory cytokine IFN-γ controls IL-10 production by B cells but cytokines regulating IL-10 production by T cells suppress the regulatory functions of B cells.

It has been shown that HIF-1α could be a critical transcription factor for IL-10 production by B cells in autoimmune diseases [15]. We have shown that IFN-γ increased the expression of *HIF-1α* gene similarly to the expression of *IL-10* gene in B cells. IL-4 and TGF-β did not have a significant effect on HIF-1α expression. Nevertheless, we tested also a possible role of *T*-cell transcription factors in the regulation of IL-10 production by B cells. The transcription factor GATA-3 has been identified as a crucial factor regulating IL-10 production by activated Th2 cells [18,23]. Therefore, we tested the possibility of GATA-3 expression in activated B cells and its possible role in IL-10 production by B cells. We found that activated B cells did not express GATA-3 and the expression of GATA-3 was not induced even in the presence of IFN-γ, IL-4 or TGF-β. This suggests that IL-10 production by activated B cells is independent of GATA-3 expression, in contrast to case of Th2 cells, where GATA-3 plays a key role in transcription of the *Il-10* gene [18]. Also, our tests showed that the production of IL-10 by T cells correlated with the expression of GATA-3. We observed that all IL-10^+ T cells were simultaneously GATA-3^+. On the contrary, the production of IL-10 by B cells was not dependent on GATA-3 expression. In agreement with the findings of Wang et al. [24] who showed that freshly isolated B cells do not express GATA-3, we were not able to detect GATA-3 even in activated IL-10-producing B cells.

The transcription factor FoxP3 has been described as the most

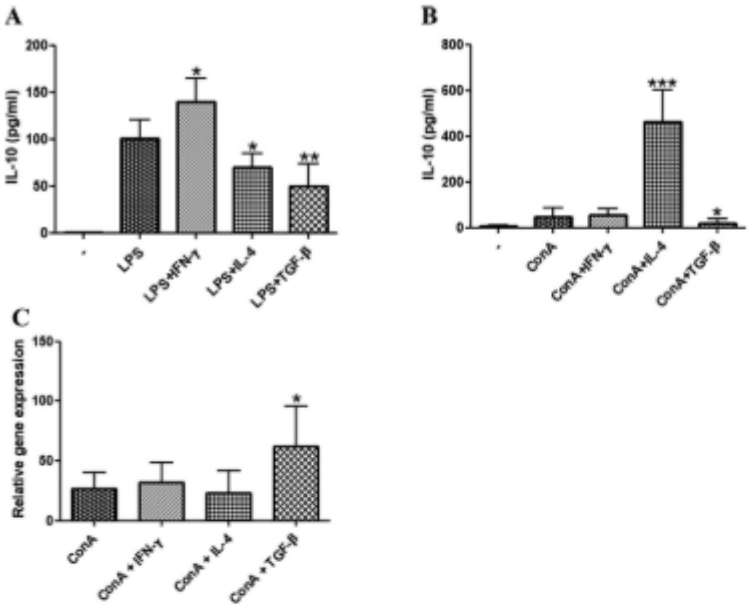

Fig. 5. The effect of IFN-γ, IL-4 and TGF-β on IL-10 production by B and T cells and expression of gene for IL-10Ra in T cells. Purified B (A) and T (B) cells were cultured for 72 h unstimulated (−) or stimulated with mitogens in the absence or presence of IFN-γ, IL-4 or TGF-β. The concentrations of IL-10 in the supernatants were determined by ELISA. Purified T cells (C) were stimulated for 48 h with Con A in the absence or presence of IFN-γ, IL-4 or TGF-β. The expression of gene for IL-10Ra was determined by real-time PCR. Each bar represents the mean + SD from four independent determinations. Values with asterisks are significantly different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) from the control (the cells stimulated with mitogen in the absence of added cytokines).

important factor for Treg development and their functions [10,25]. Therefore, we tested the possibility of FoxP3 expression in IL-10-producing B cells. Similarly to the absence of GATA-3 expression in B cells, Bregs in our model do not express detectable level of Foxp3, and this transcription factor was not induced in B cells even in the presence of IFN-γ, which significantly elevated IL-10 production. Contrary to our findings, some authors have suggested that Bregs can express FoxP3 in both humans and mice. The presence of FoxP3$^+$ B cells has been described in models of cow's milk allergy, autoimmune arthritis and systemic lupus erythematosus [26–28]. FoxP3 has also been detected in some tumor cells [29,30] and in macrophages [31,32]. However, the presence of FoxP3$^+$ Bregs has remained unclear and controversial, and it is still a matter of discussion [14]. One major difference between our study, demonstrating the absence of GATA-3 and FoxP3 expression in Bregs, and those of other authors who have described the expression of GATA-3 and FoxP3 in Bregs, could be our use of Alexa Fluor 488-conjugated anti-GATA-3 and anti-FoxP3 mAb to analyze GATA-3 or FoxP3 expression. In our hands, PE-conjugated anti-FoxP3 mAb which were used in the majority of other studies, provided a false-positive staining. It also cannot be excluded that different results arise from the use of different experimental models, activation signals and detection techniques and one of limitations could be a use of whole B- and T-cell populations. We confirmed the absence of GATA-3 and FoxP3 expression in IL-10-producing B cells, in comparison to T cells, also at the level of gene expression using real-time PCR.

In summary, our results have shown that the antagonistic cytokines IFN-γ, IL-4 and TGF-β, which are involved in T-cell subset development, regulate IL-10 production by activated B and T cells in different ways. While the production of IL-10 by activated T cells is increased in the presence of IL-4 or TGF-β, these cytokines rather inhibit IL-10 production by B cells. IFN-γ has no effect on IL-10 production by T cells, but significantly elevates the production of IL-10 by activated B cells. In addition, while IL-10 production by T cells requires GATA-3 expression, and FoxP3 is indispensable for Treg functions, the production of IL-10 by B cells is independent of the presence of GATA-3 or FoxP3.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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4.4. Difference between mitogen-stimulated B and T cells in nonspecific binding of R-PE-conjugated antibodies

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Flow cytometry analysis is a powerful tool for the immunophenotyping of cells. It plays a role in the determination of individual cell populations, but it is essential to preciously distinguish between accurate staining and false positive or negative staining. Nonspecific binding of conjugated antibodies is a critical issue that could dramatically influence flow cytometry analysis accuracy. Nonspecific binding can affect only particular staining procedures and cell types. In this study, we analyzed the nonspecific binding of R-phycoerythrin (R-PE)-conjugated antibodies to mouse LPS-stimulated B cells, in contrast to ConA-stimulated T cells.

Mitogen-stimulated lymphocytes were fixed, permeabilized, and stained using isotype control antibodies conjugated with fluorescein isothiocyanate (FITC), allophycocyanin (APC), R-PE, or AlexaFluor 488 fluorochromes and assessed by flow cytometry. R-PE-conjugated antibodies exerted nonspecific binding, these antibodies bound to LPS-stimulated B cells. According to the used isotype control antibodies or the fixation/permeabilization kit, the percentage of R-PE positive B cells varied. Nevertheless, up to 30% of $CD19^+$ B cells were R-PE positive when R-PE-conjugated isotype control antibodies were used. This binding of R-PE-conjugated antibodies to LPS-stimulated B cells did not depend on the haplotype of mouse strain. We also analyzed the staining of mitogen-stimulated B and T cells by unconjugated R-PE molecules. The R-PE positivity of B cells was in a dose-dependent manner. ConA-stimulated T cells slightly bound R-PE molecules only in the highest concentrations.

The data demonstrated that LPS-stimulated B cells, in contrast to ConA-stimulated T cells, bind R-PE molecules nonspecifically to intracellular structures following formaldehyde or paraformaldehyde fixation. Altogether, the results based on the use of R-PE-conjugated antibodies for intracellular marker staining of activated B cells must be taken with precaution.

Aspirant's contribution: sample analyses, data analyses and interpretation, manuscript writing.

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Difference between mitogen-stimulated B and T cells in nonspecific binding of R-phycoerythrin-conjugated antibodies

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ABSTRACT

Nonspecific binding of conjugated antibodies represents a critical step which could significantly influence the results of immunostaining or flow cytometry. In this respect, various staining procedures and distinct cell types can alter the results obtained with different fluorochromes. In this study, we analysed nonspecific binding of R-phycoerythrin (R-PE)-conjugated antibodies to mouse mitogen-stimulated B and T lymphocytes. The cells were fixed, permeabilized and stained using isotype control antibodies conjugated with different fluorochromes and assessed by flow cytometry. R-PE-conjugated antibodies bound to LPS-stimulated B cells, in contrast to Con A-stimulated T cells, independently of their specificity. The percentage of R-PE positive B cells varied, according to the used antibodies or the fixation/permeabilization kit. Nevertheless, up to 30% of R-PE$^+$ B cells after staining with R-PE-conjugated isotype control antibodies was detected. Furthermore, LPS-stimulated B cells bound nonspecifically, in a dose-dependent manner, unconjugated R-PE molecules. Con A-stimulated T cells slightly bound R-PE only in high concentrations. Similarly, the antibodies conjugated with other fluorochromes showed less than 1% of nonspecific binding independently of the manufacturer of antibodies or fixation/permeabilization kits. The data demonstrated that LPS-stimulated B cells, in contrast to Con A-stimulated T cells, bind R-PE nonspecifically following formaldehyde or paraformaldehyde fixation. Therefore, the results based on the use of R-PE-conjugated antibodies should be taken with a precaution.

1. Introduction

Flow cytometry represents a powerful tool to quickly distinguish between the antigen-positive and antigen-negative populations of cells, based on detection of surface or intracellular markers. In clinical practice, it plays an important role in medicine diagnosis. Therefore, it is necessary to exactly control the specificity of measurement to avoid false-positive or false-negative results.

The determination of intracellular markers requires a fixation and permeabilization of cells to enable the access of antibodies to the epitopes of antigen. For this purpose, a fixation of cells by formaldehyde or paraformaldehyde, a polymeric version of formaldehyde, is widely used (Kiernan, 2000). The mechanism of action of these fixation reagents is based on the formation of intra- and inter-molecular cross-links among cell structures (Howat and Wilson, 2014). However, fixation can modify molecular epitopes and cell characteristics (Stewart et al., 2007), and thus it is important to avoid of the nonspecific binding of antibodies as an output of flow cytometry analysis. A further key step of flow cytometric protocol is the choice of fluorochromes with suitable characteristics for the selected system, such as excitation and emission profiles, relative brightness, photostability or their compatibility with the configuration of a flow cytometer (Flores-Montero et al., 2019). The fluorochromes widely used in flow cytometry include fluorescein isothiocyanate (FITC), R-phycoerythrin (R-PE) or allophycocyanin (APC).

R-PE belongs to the phycobiliproteins which represent a family of light-harvesting macromolecules and are the components of photosynthetic apparatus in cyanobacteria, red algae and cryptomonads (Apt et al., 1995). R-PE is a large protein complex with highly fluorescent

Abbreviations: APC, allophycocyanin; Con A, concanavalin A; DAPI, 4′,6′-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; mAb, monoclonal antibodies; R-PE, R-phycoerythrin..

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attributes (Batard et al., 2002). As a result of large Stokes shift and brightness, R-PE is widely used for conjugation with specific monoclonal or polyclonal antibodies in flow cytometry (Onlamoon et al., 2011). However, R-PE represents large pigment-protein complexes (Wang et al., 2015) which could bind to low affinity FcγRII and FcγRIII receptors for monomeric IgG antibodies expressed particularly on myeloid cells (Takizawa et al., 1993; Bournazos et al., 2016). Nevertheless, in standard protocols for flow cytometry staining it is usually solved using anti-CD16/CD32 antibodies or using a serum to block FcγRII and FcγRIII receptors.

In the present study, we analysed a difference between nonspecific binding of R-PE-conjugated antibodies to mitogen-stimulated B and T cells. R-PE-conjugated antibodies are bound to lipopolysaccharide (LPS)-stimulated mouse B cells, in contrast to concanavalin A (Con A)-stimulated T cells, after formaldehyde or paraformaldehyde fixation and permeabilization. In addition, R-PE itself bound nonspecifically to intracellular structures of activated B cells. These data showed that the use of R-PE-conjugated antibodies for the staining of intracellular markers in LPS-stimulated B cells can be influenced by a nonspecific binding of R-PE, and this binding can cause false-positive results. On the other hand, the use of R-PE-conjugated antibodies for the staining of markers in activated T cells does not influence the results of flow cytometry.

2. Materials and methods

2.1. Animals

Female mice of inbred strains BALB/c or C57BL/6 were purchased from the breeding unit of the Institute of Molecular Genetics of the Czech Academy of Sciences, Prague. The use of the animals was approved by the Local Ethical Committee of the Institute of Experimental Medicine.

2.2. B-cell and T-cell enrichment

Single cell suspensions of spleen cells were prepared in a complete RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (Gibco BRL, Grand Island, NY, USA), antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, Sigma-Aldrich), 10 mM HEPES buffer (Sigma-Aldrich) and 5×10^{-5} M 2-mercaptoethanol (Serva, Heidelberg, Germany). To isolate the B-cell population, spleen cell suspension was incubated for 15 min with CD19 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. B cells were isolated by a positive selection using a magnetic-activated cell sorter (MACS, AutoMACS, Miltenyi Biotec). CD19-negative cells obtained after isolation of B cells were used as T-cell-enriched population. This T-cell population contained approximately 65% of $CD3^+$ T cells.

2.3. The characterization of isolated B-cell and T-cell populations

To characterize the purity and phenotype of the enriched B-cell population, the cells were incubated for 30 min at 4 °C with the following monoclonal antibodies (mAb): FITC-conjugated anti-CD19 (clone 6D5), APC-conjugated anti-CD3 (clone 17A2) and R-PE-conjugated anti-F4/80 (clone BM8). The phenotype of the enriched T-cell population was characterized using the following mAb: APC-conjugated anti-CD3 (clone 17A2), FITC-conjugated anti-CD19 (clone 6D5) and R-PE-conjugated anti-F4/80 (clone BM8). All antibodies were purchased from BioLegend (San Diego, CA, USA). The dead cells were stained by adding Hoechst 33258 fluorescent dye (Invitrogen, Carlsbad, CA. USA) to the samples 15 min before flow cytometry analysis. Data were collected by LSR II cytometer (BD Biosciences, Franklin Lakes, NJ, USA), 50,000 live and single cells were recorded. Data were analysed using FlowJo software (LLC, Ashland, OR. USA), within displayed events, only viable and single cells were gated and all gates were set based on FMO controls.

Table 1.
List of used antibodies for intracellular staining.

Antibody	Clone	Fluorochrome	Used concentration (μg/mL)	Manufacturer
rat IgG2bκ isotype control	eB149/10H5	FITC	4	eBioscience
rat IgG2bκ isotype control	eB149/10H5	APC	4	eBioscience
rat IgG2bκ isotype control	eB149/10H5	R-PE	4	eBioscience
rat Foxp3 (IgG2aκ isotype)	FJK-16 s	R-PE	4	eBioscience
mouse Foxp3 (IgG1κ isotype)	150D/E4	Alexa Fluor 488	4	eBioscience
rat GATA3 (IgG2bκ isotype)	TWAJ	R-PE	0.24	eBioscience
rat GATA3 (IgG2bκ isotype)	TWAJ	Alexa Fluor 488	0.24 or 1	eBioscience
mouse IgG2bκ isotype control	27–35	FITC	4	BioLegend
mouse IgG2bκ isotype control	27–35	APC	4	BioLegend
mouse IgG2bκ isotype control	27–35	R-PE	4	BioLegend
rat IgG2bκ isotype control	RTK4530	R-PE	4	BioLegend
rat IgG2bκ isotype control	A95-1	R-PE	2 or 4	BD Pharmingen

2.4. Flow cytometry analysis

Purified B cells at a concentration of 1×10^6 cells/mL or enriched T-cell population at a concentration of 0.5×10^6 cells/mL, were incubated in 48-well tissue culture plates (Corning Inc., Corning, NY, USA) in a final volume of 1 mL of complete RPMI-1640 medium stimulated by B-cell mitogen LPS from *Escherichia coli* 0127:B8 (10 μg/mL, Sigma-Aldrich) or T-cell mitogen concanavalin A (Con A, 1 μg/mL, Sigma-Aldrich) for 72 h. For kinetic analysis, the cells were incubated for 24, 48 or 72 h.

After incubation, the cells were collected and incubated with anti-CD16/CD32 mAb (Invitrogen) against mouse FcγII/III receptors at an amount of 0.5 μg per 10^6 cells at 4 °C for 20 min and twice washed by PBS. The dead cells were stained with a Zombie Violet Fixable Viability Kit (1:500; BioLegend) before intracellular staining. The cells were

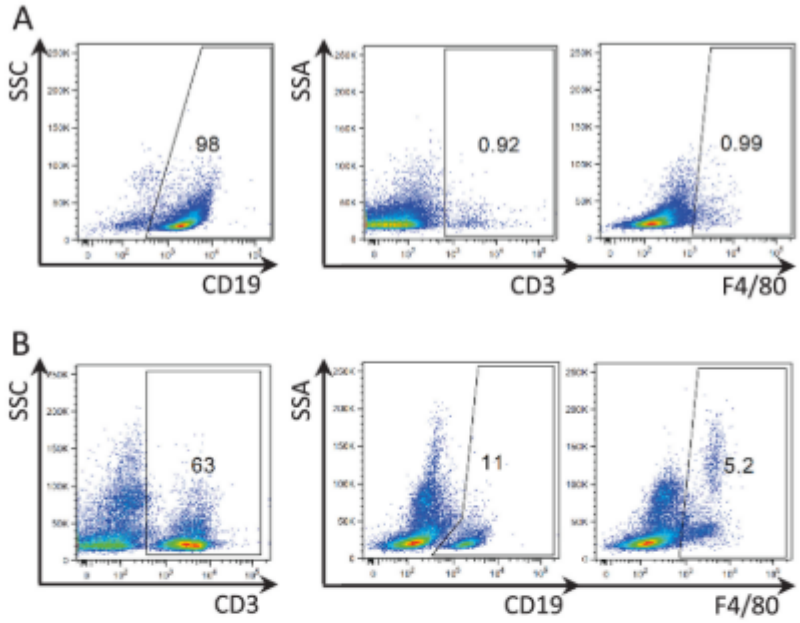


Fig. 1. The phenotypic characterization of enriched B-cell (A) and T-cell (B) populations. The cells were separated using a magnetic-activated cell sorting and their phenotype was determined by flow cytometry. Representative dot plots indicate the percentage of $CD19^+$, $CD3^+$ and $F4/80^+$ cells among the B-cell population, and the percentage of $CD3^+$, $CD19^+$ and $F4/80^+$ cells among the T-cell population. One typical experiment from three individual measurements is shown.

stained with R-PE- or FITC-conjugated anti-CD19 mAb (clone 6D5, BioLegend) or R-PE- or FITC-conjugated anti-CD3 mAb (clone 17A2, BioLegend) and then twice washed by PBS, fixed and permeabilized. Three different sets were used for fixation and permeabilization. The samples were fixed in 100 µL of Foxp3 staining buffer sets from eBioscience (San Diego, CA) at 4 °C for 30 min, in 100 µl of Foxp3 Fix/Perm buffer set from BioLegend at room temperature for 20 min or in 100 µl of Foxp3 buffer set from BD Pharmingen at 4 °C for 40 min. All fixation setups were according to the manufacturer's instructions. Then samples were washed by appropriate permeabilization buffer and stained intracellularly at 4 °C for 30 min, a list of used mAb is summarized in Table 1 and then twice washed by appropriate permeabilization buffer. To characterize the role of R-PE unconjugated with antibodies, the samples were incubated at 4 °C for 30 min with R-PE (Invitrogen) at an amount of 0.007, 0.07, 0.7, 7, 70, and 700 ng R-PE per 10^6 cells. The concentration 4 µg/mL of most diluted antibodies corresponds to 25 ng R-PE (F/P ratio = 1).

The samples were measured in 200 µL of PBS and analysed using the LSRII flow cytometer (BD Bioscience). All flow cytometry data were analysed using FlowJo software (LLC). Gates were placed around lymphocytes in a FSC/SSC plot, next the live population was identified based on Zombie Violet Fixable Viability Kit, subsequently the doublets were excluded using a FSC-A/FSC-H plot, the $CD19^+$ or $CD3^+$ population was then identified and then the population of FITC-, APC- or R-PE-positive cells was gated.

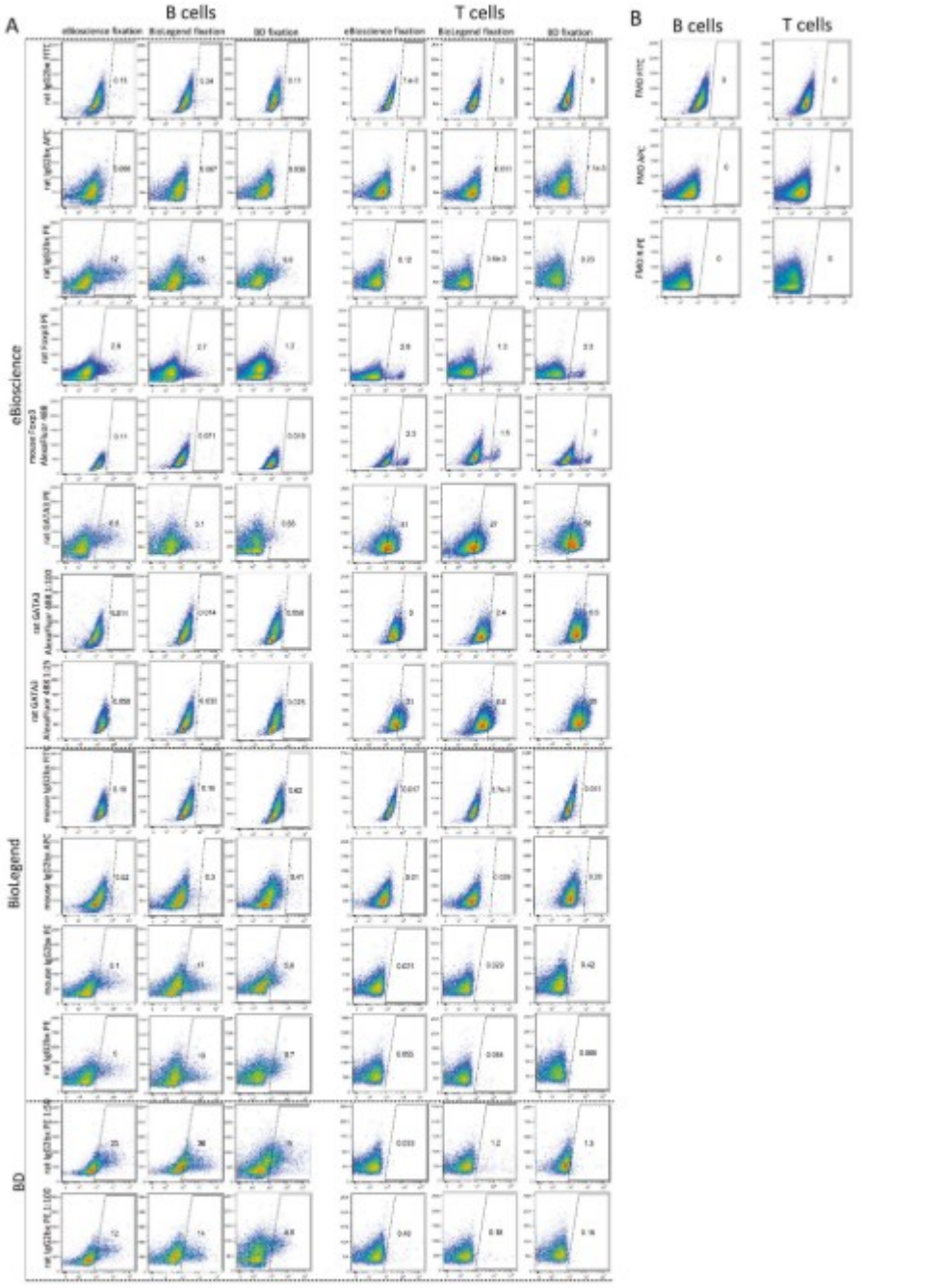
2.5. Microscopy

The isolated B-cell population at a concentration of 1×10^6 cells/mL, was stimulated with LPS (10 µg/mL) for 72 h. The cells were stained with FITC-conjugated anti-CD19 mAb (clone 6D5, BioLegend) at 4 °C for 30 min and then fixed and permeabilized using a Foxp3 Staining Buffer Set (eBioscience), according to the manufacturer's instructions. The samples were stained intracellularly with R-PE-conjugated anti-GATA3 mAb (clone TWAJ, eBioscience) at 4 °C for 30 min. A smear sample was prepared from stained cells. The samples were fixed on glass slides with Mowiol 4–88 (Calbiochem, San Diego, CA, USA) in the presence of the nuclear dye 4′,6′-diamidino-2-phenylindole (DAPI). Visualization of the fluorescent label was performed using a fluorescent microscope (Leica, Wetzlar, Germany) and analysed using ImageJ software.

3. Results

3.1. Phenotypic characterization of purified B-cell and T-cell populations

Purified spleen B-cell and T-cell populations were phenotypically characterized by flow cytometry. As demonstrated in Fig. 1, the isolated B-cell population contained about 98% of $CD19^+$ cells. This population included less than 1% of $CD3^+$ or $F4/80^+$ cells (Fig. 1A). The enriched T-cell population contained more than 60% of $CD3^+$ cells, and about 10% of cells were $CD19^+$ and approximately 5% of cells were $F4/80^+$ (Fig. 1B).



(caption on next page)

Fig. 2. Representative dot plots demonstrating the percentage of fluorochrome positive B and T cells after different fixation/permeabilization protocol and staining with isotype controls. Purified B cells and enriched T cells from BALB/c mice were stimulated with LPS or Con A for 72 h. The percentage of $FITC^+CD19^+$, Alexa Fluor 488^+CD19^+, APC^+CD19^+ and $R\text{-}PE^+CD19^+$ cells or $FITC^+CD3^+$, Alexa Fluor 488^+CD3^+, APC^+CD3^+ and $R\text{-}PE^+CD3^+$ cells was determined by intracellular staining using different fixation reagents for flow cytometry. X-axis of dot plots represents SSC, y-axis of dot plots represents intensity of fluorescence signal. One representative dot plot from four independent experiments is shown.

3.2. The comparison of nonspecific antibody-binding to BALB/c mouse splenocytes in different fixation and permeabilization conditions

The flow cytometry analysis of LPS-stimulated B cells of BALB/c origin using R-PE-conjugated Foxp3 and GATA3 antibodies revealed a population of Foxp3- and GATA3-expressing B cells, but the antibodies of the same isotype conjugated with Alexa Fluor 488 did not show staining for Foxp3 and GATA3 transcription factors. Using of R-PE- and Alexa Fluor 488-conjugated Foxp3 and GATA3 antibodies to measure Con A-stimulated T cells led to comparable results. To solved this discrepancy, we used a panel of isotype control antibodies conjugated with different fluorochromes. Nevertheless, similarly to the R-PE-conjugated Foxp3 and GATA3 antibodies, the B-cells population was also stained with R-PE-conjugated isotype control antibodies. Therefore, we tested the specificity of used antibodies and intracellular staining protocols.

The rat and mouse IgG2bκ isotype control antibodies conjugated with different fluorochromes from three different manufacturers were used for the intracellular staining of mitogen-stimulated B and T cells. We also compared different fixation/permeabilization kits from distinct manufacturers. The B or T cells were stimulated for 72 h with LPS or Con A, fixed, permeabilized, stained with fluorochrome-conjugated antibodies and analysed by flow cytometry. Fig. 2 shows representative dot plots from intracellular staining with anti-mouse Foxp3 antibody conjugated with R-PE or Alexa Fluor 488, anti-mouse GATA3 antibody conjugated with R-PE or Alexa Fluor 488 or with rat or mouse IgG2bκ isotype control antibodies conjugated with different fluorochromes. The mouse and rat isotype control antibodies conjugated with FITC or APC showed less than 1.5% of nonspecific binding to both B and T cells, independently of the manufacturer or fixation/permeabilization kit. Similarly, anti-mouse Foxp3 or GATA3 antibodies conjugated with Alexa Fluor 488 nonspecifically bound to less than 1% of LPS-stimulated B cells. On the other hand, R-PE-conjugated isotype control antibodies and R-PE-conjugated Foxp3 or GATA3 antibodies stained a significant proportion of activated B cells. The percentage of positive B cells slightly varied according to the used isotype control antibodies or fixation/permeabilization kit, but a proportion of R-PE-positive B cells reached up to 30% (Fig. 3). R-PE-conjugated antibodies bound to LPS-stimulated B cells independently of antibody specificity (Fig. 3A, C, E). Con A-stimulated T cells were only positive after staining with anti-mouse Foxp3 or GATA3 antibodies conjugated with both R-PE and Alexa Fluor 488 (Fig. 3B, D, F). The percentage of positive T cells only differed in dependence on a titre of antibodies.

To test whether the observed nonspecific staining of activated B cells only occurred in the flow cytometric analysis, or more generally, we performed immunohistochemistry staining using anti-mouse R-PE-conjugated GATA3 antibodies to study in more detail the binding of these antibodies to LPS-stimulated B cells. As demonstrated in Fig. 4, R-PE-conjugated antibodies nonspecifically bound to the structures of activated B cells. Altogether, the results presented in Figs. 2–4 show that R-PE-conjugated antibodies bound nonspecifically to LPS-stimulated B cells.

3.3. Nonspecific antibody binding to C57BL/6 mouse splenocytes in different fixation and permeabilization protocols

To confirm that nonspecific binding of R-PE-conjugated antibodies is not only restricted to B cells of the BALB/c strain, we next analysed B cells of C57BL/6 origin. Fig. 5 shows the proportion of fluorochrome-positive $CD19^+$ B and $CD3^+$ T cells from C57BL/6 mice. Similarly to cells of BALB/c origin, mouse and rat isotype control antibodies, conjugated with FITC or APC, showed less than 2% of nonspecific antibody binding to both B and T cells independently of the manufacturer of antibodies or a fixation/permeabilization kit. Less than 0.5% of LPS-stimulated B cells were positive after staining with Alexa Fluor 488-conjugated GATA3 antibodies. On the other hand, LPS-stimulated B cells were stained with R-PE-conjugated isotype control antibodies or R-PE-conjugated GATA3 antibodies. The percentage of R-PE positive $CD19^+$ B cells varied, according to the used isotype control antibodies or fixation/permeabilization kit, but was independent on the antibody specificity (Fig. 5A, C, E). Con A-stimulated T cells were only positive with anti-mouse GATA3 antibodies conjugated with both R-PE and Alexa Fluor 488 (Fig. 5B, D, F). These results demonstrated that R-PE-conjugated antibodies bound nonspecifically to LPS-stimulated B cells, independently of the mouse strain.

3.4. The time dependence of nonspecific binding of R-PE-conjugated antibodies to activated B cells

To test the time dependency of nonspecific binding of R-PE-conjugated antibodies on the activation state of B cells, the percentage of $R\text{-}PE^+$ B cells was determined after 24, 48 and 72 h of LPS stimulation. Nonspecific binding of R-PE-conjugated rat IgG2bκ isotype control antibodies increased over time, independently of the manufacturer of antibodies or fixation/permeabilization kit (Fig. 6A, B, C). There was only a minimal binding of R-PE-conjugated antibodies to unstimulated cells and the binding progressively increased with cell activation. On the contrary to activated B cells, there was only a marginal nonspecific binding of antibodies to fresh B cells (Fig. 6D) or B cells which were cultured for 72 h unstimulated (Fig. 6E). The data showed that nonspecific binding of R-PE-conjugated antibodies was dependent on the activation of B cells.

3.5. Nonspecific binding of R-PE molecules to activated B and T cells

To verify the possibility that nonspecific binding of R-PE-conjugated antibodies to activated B cells could be caused by a binding of fluorescent molecules themselves and not by nonspecific binding of antibodies, we tested the binding of unconjugated R-PE molecules instead of R-PE-conjugated antibodies. The purified $CD19^+$ B cells and enriched $CD3^+$ T cells were stimulated for 72 h with LPS or Con A respectively; the cells were treated according to the protocol for intracellular staining and the antibodies in the intracellular staining step were replaced by the unconjugated R-PE used in different amounts. Fig. 7 shows that the binding of R-PE molecules to LPS-stimulated B cells was dose-dependent in all fixation/permeabilization systems. Con A-stimulated T cells slightly

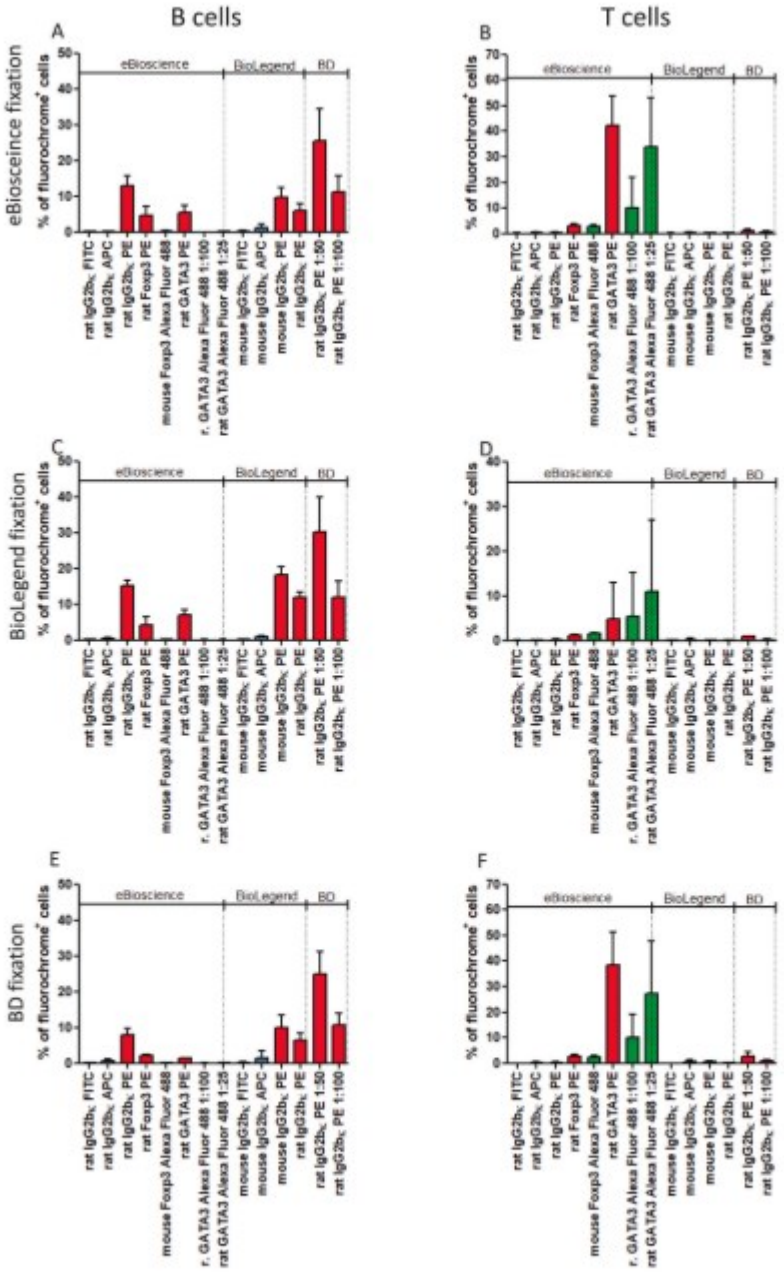


Fig. 3. Quantitative comparison of the percentage of fluorochrome positive B and T cells from BALB/c mice after different fixation/permeabilization protocol and staining with isotype controls. Purified $CD19^+$ B cells (A,C,E) and enriched $CD3^+$ T cells (B,D,F) from BALB/c mice were stimulated with LPS or Con A for 72 h, and the cells were prepared for intracellular flow cytometry. The cells were fixed with eBioscience (A,B), BioLegend (C,D) or BD (E,F) fixation reagent. Each bar represents the mean + SD from four independent experiments which were performed in singlet layout.

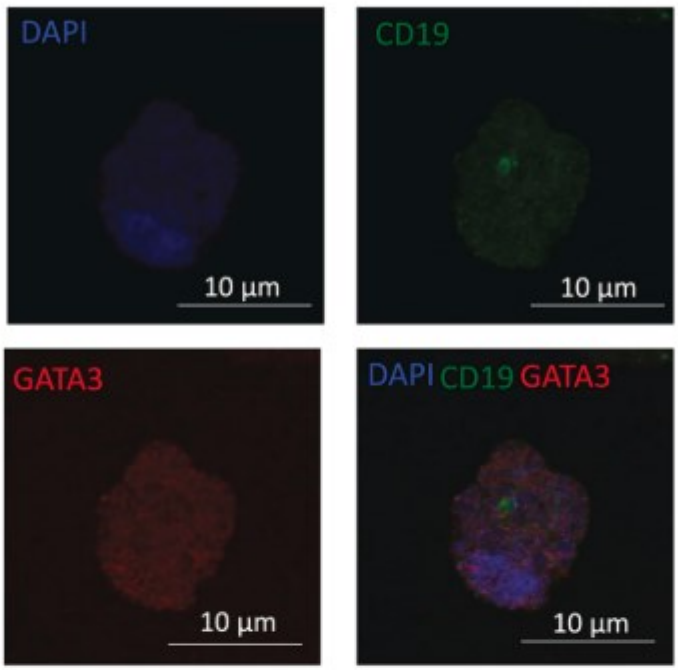


Fig. 4. Fluorescent immunocytochemistry of LPS-stimulated B cells. Purified B cells from BALB/c mice were stimulated for 72 hs with LPS, stained with FITC-conjugated CD19 antibodies (green), R-PE-conjugated GATA3 antibodies (red) and DAPI (blue). Images were overlaid with the indicated colours on top of each image. Scale bars in white, 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bound R-PE in high concentrations in eBioscience and BD fixation systems (Fig. 7B, F). Taken together, these data indicate that unconjugated R-PE molecules are bound to intracellular structures of LPS-stimulated B cells and can cause false-positive results and impair flow cytometry analysis.

4. Discussion

Flow cytometry analysis represents a helpful tool for cell immunophenotyping and therefore plays an important role in the determination of individual cell populations. In this respect it is necessary to exactly distinguish between the real staining and a false positive or negative staining. The staining of intracellular markers is even more complicated because it is necessary to fix and permeabilize cells to make intracellular markers accessible for antibodies. However, this process might change epitopes and cell characteristics (Stewart et al., 2007) and therefore it is important to choose appropriate fluorescent probes. One of the widely used fluorescent proteins for flow cytometry is R-PE. Although R-PE is a large protein complex, it shows excellent fluorescent attributes, such as large Stokes shift and brightness (Batard et al., 2002; Onlamoon et al., 2011). In spite of these beneficial properties, the nonspecific binding of R-PE complexes to intracellular structures of activated B cells has been recorded (Takizawa et al., 1993; Tabary et al., 2008; Kim and Kim, 2013).

In this study, we demonstrated that a nonspecific binding of R-PE-conjugated antibodies to murine LPS-stimulated B cells occurred independently of antibody specificity or isotype during an intracellular staining. Flow cytometry analysis using R-PE-conjugated Foxp3 and GATA3 antibodies revealed a population of B cells which expressed T cell lineage-specific transcription factors Foxp3 or GATA3. However, the population of $Foxp3^+$ or $GATA3^+$ B cells was not detected when the same isotype antibodies conjugated with Alexa Fluor 488 fluorescent dye were used. To analyse this discrepancy, we tested a panel of rat and mouse IgG2bκ isotype control antibodies conjugated with FITC, APC or R-PE purchased from different manufacturers and various fixation/permeabilization kits were also used. Murine T cells served as control cells. The results showed that nonspecific binding of FITC-, APC- and Alexa Fluor 488-conjugated antibodies to mitogen-stimulated B or T cells was negligible. However, up to 30% of $CD19^+$ LPS-stimulated B cells, in contrast to Con A-stimulated T cells, nonspecifically bound R-PE-conjugated isotype control antibodies. The binding of R-PE-conjugated antibodies to activated B cells did not depend on the haplotype of mouse strain. To exclude a possibility that R-PE-conjugated antibodies bound to LPS-stimulated B cells independently of antibodies themselves, we analysed staining of mitogen-stimulated B and T cells by unconjugated R-PE molecules. R-PE binds to LPS-activated B cells in dose-dependent manner and that this binding does not depend on fixation/permeabilization kits. Con A-stimulated T cells bound the R-PE molecule

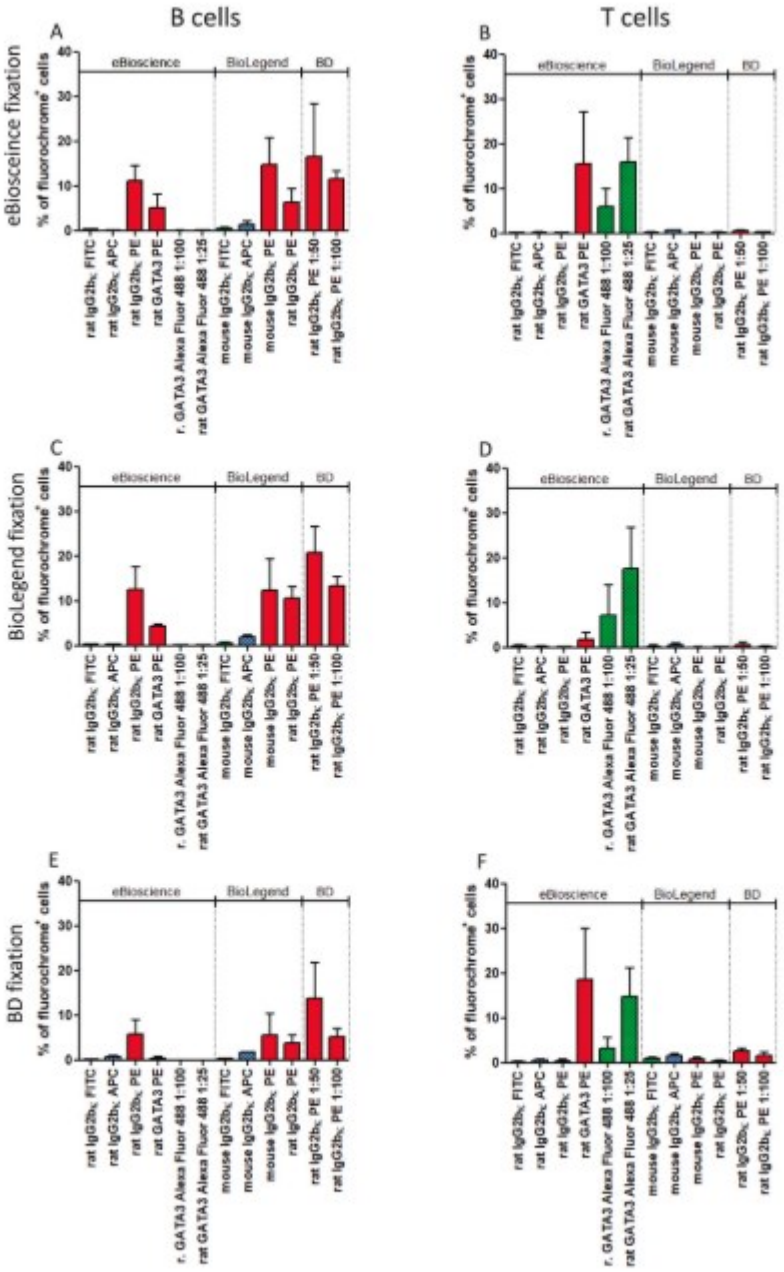


Fig. 5. The percentage of fluorochrome positive B and T cells from C57BL/6 mice after different fixation/permeabilization protocol and staining with isotype controls. Purified $CD19^+$ B cells (A,C,E) and enriched $CD3^+$ T cells (B,D,F) from C57BL/6 mice were stimulated for 72 ho with LPS or Con A prior to the cell preparation for intracellular flow cytometry. The cells were fixed with eBioscience (A,B), BioLegend (C,D) and BD (E,F) fixation reagent. Each bar represents the mean + SD from four independent determinations which were performed in singlet layout.

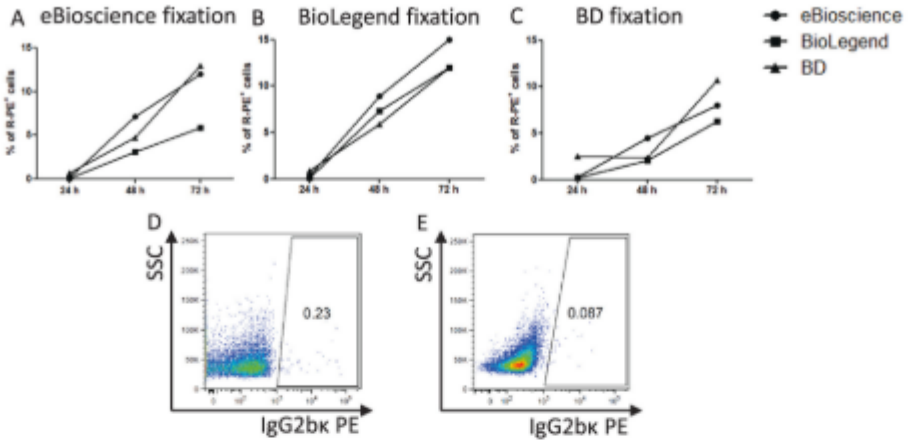


Fig. 6. Kinetics of nonspecific binding of rat PE-conjugated IgG2bκ antibody to B cells from BALB/c mice after different fixation/permeabilization protocol. Purified $CD19^+$ B cells were stimulated for 24, 48 or 72 h with LPS and the cells were stained for intracellular flow cytometry. The cells were fixed with eBioscience (A), BioLegend (B) or BD (C) fixation reagent. Freshly isolated B cells (D) or unstimulated B cells for 72 h (E) were stained for intracellular flow cytometry. Each point represents the mean or one representative dot plot from two independent determinations which were performed in singlet layout.

slightly and only in the highest concentrations of R-PE.

The possibility of nonspecific binding of R-PE in flow cytometry analysis was firstly suggested by Takizawa et al. (1993), who showed that R-PE could slightly bind to FcγRII and FcγRIII receptors of myeloid cells. This nonspecific binding of R-PE molecules was abrogated by antibodies blocking the FcγRII and FcγRIII receptors prior to flow cytometry staining. It has been also shown that R-PE molecules conjugated with antibodies caused false-positive intracellular staining of cells which did not express FcγRII and FcγRIII receptors (Tabary et al., 2008; Kim and Kim, 2013). To extend the above results, we showed that R-PE molecules themselves bind to murine LPS-stimulated B cells, but not to Con A-stimulated T cells. Our data showed that LPS-stimulated B cells do not express transcription factors Foxp3 and GATA3, as we have described previously using Alexa Fluor 488-conjugated anti-Foxp3 and anti-GATA3 antibodies (Bohacova et al., 2020). Contrary to our findings, some authors have suggested that B cells can express T cell lineage-specific transcription factors, such as Foxp3 (Noh et al., 2012; Vadasz et al., 2015; Park et al., 2016; Vadasz and Toubi, 2017). The main difference between our study and the results of other authors (Noh et al., 2012; Vadasz et al., 2015; Park et al., 2016; Vadasz and Toubi, 2017) was the use of Alexa Fluor 488-conjugated anti-Foxp3 and anti-GATA3 antibodies in our study (Bohacova et al., 2020), while R-PE-conjugated antibodies were used by other authors. Park et al. (2016) used additional methods, not only fluorescence-based techniques, to prove Foxp3 expression in B cells. These authors used for isolation of $CD19^+$ lymphocytes the MACS separation columns yielding maximally 90% of purity based on our experience. The other methods used for detection of Foxp3, such as western blotting or RT-PCR, do not allow to exclude the effect of contaminated T cells which could be responsible for false-positive results. Therefore, other more sensitive techniques are required for a definitive confirmation of expression or absence of T cell transcriptional factors in B cells. As we have demonstrated here, R-PE-conjugated antibodies could provide false-positive results during intracellular staining of stimulated B cells, and thus the results obtained with R-PE-conjugated antibodies should be taken with a precaution. More complex verification of binding specificity of tandem dyes containing R-PE will be necessary.

In summary, our results have shown that murine LPS-stimulated B cells, in contrast to Con A-stimulated T cells, nonspecifically bind R-PE-conjugated antibodies after formaldehyde or paraformaldehyde fixation. Furthermore, we have demonstrated that B cells bound R-PE molecules which are not conjugated with antibodies. The results have shown that the staining of intracellular markers in LPS-stimulated B cells by R-PE-conjugated antibodies influences the staining specificity and accuracy. Therefore, it is necessary to avoid R-PE-conjugated antibodies for intracellular characterization of activated B cells because R-PE fluorochrome can cause detection of a false-positive population and thus have serious impacts on the flow cytometry analysis.

Authors contributions

P.B. and E.J. designed the experiments. P.B., M.H., B.H. and E.J. performer the flow cytometry experiments and analysed the results. J.K. performed the microscopy experiment and analysis. P.B. and V.H. wrote the manuscript.

Declaration of Competing Interest

The authors have no financial or commercial conflicts of interest.

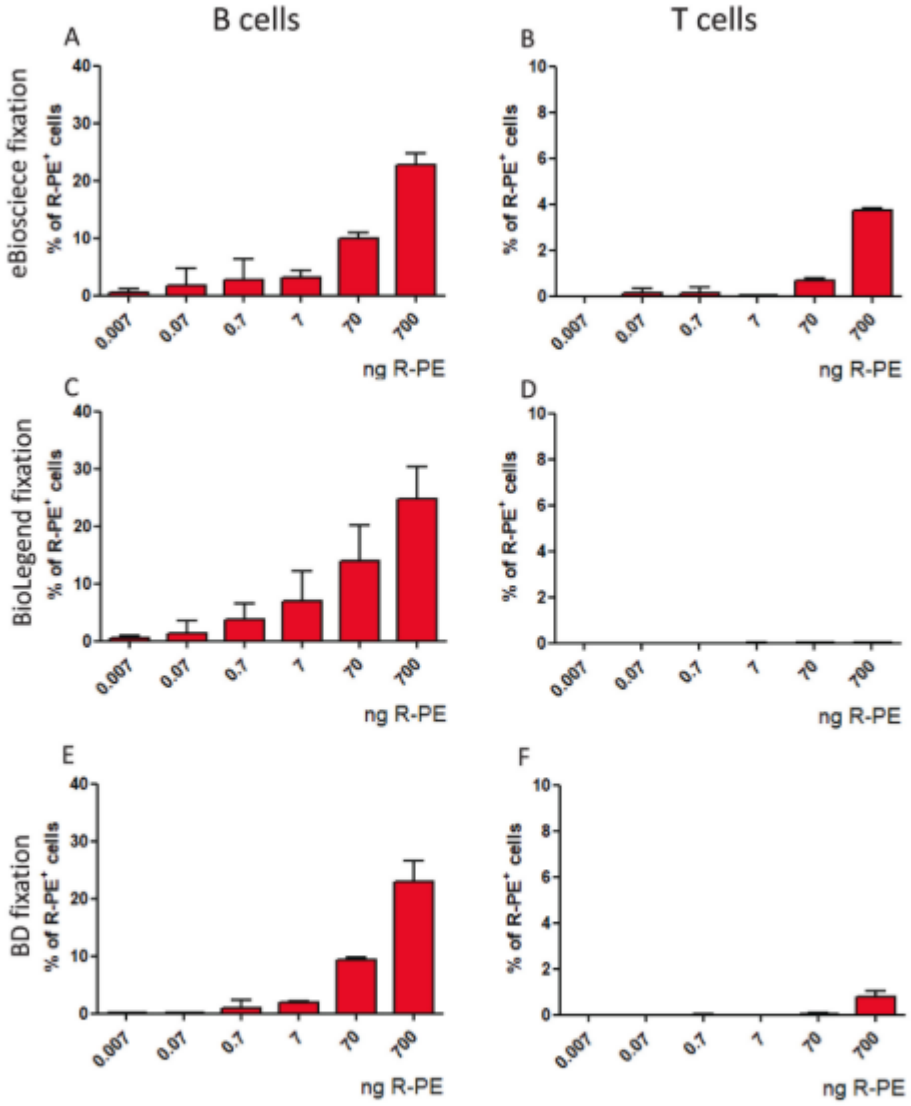


Fig. 7. The proportion of R-PE$^+$ B and T cells from BALB/c mice after different fixation/permeabilization protocol and staining with R-PE. Purified CD19$^+$ B cells (A, C,E) and enriched CD3$^+$ T cells (B,D,F) from BALB/c mice were stimulated for 72 h with LPS or Con A and stained for intracellular flow cytometry. The cells were fixed by eBioscience (A,B), BioLegend (C,D) or BD (E,F) fixation reagent and incubated with different concentrations of R-PE. Each bar represents the mean + SD from three independent determinations which were performed in singlet layout.

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5. Discussion

Regulation of the immune reactions comprises an entire system of maintaining physiological homeostasis, self-tolerance, and host defense. It is crucial to regulate immune reactions to avoid adverse tissue damage. In this respect, effector immune cells possess potent mechanisms for the elimination of treads for body integrity. Even though comprehensive research, the understanding of immunoregulation remains incomplete. Detailed insights into molecular mechanisms of immunomodulation can offer novel approaches for specific and personalized treatment of autoimmune diseases, transplantation reactions, or cancer. Thus, in the presented work, we focused on different ways of induction, expansion, and activation of immunoregulatory cells with a focus on Tregs and Bregs.

As we have shown before, the application of MSCs in combination with immunosuppressive drugs modulates inflammatory reactions in skin transplantation models (Hajkova et al., 2017b). Thus, here we extended the analysis to determine the combined effects of MSCs and immunosuppressive drugs on modulation of immune reactions in more detail. As it has been described, immunosuppressive drugs can affect MSC characteristics and immunomodulatory properties (Buron et al., 2009; Kim et al., 2015; Lightner et al., 2019). Therefore, we focused on investigating synergism between MSCs and immunosuppressants in the polarization of T cells *in vitro*. We selected five widely used immunosuppressive drugs with distinct action mechanisms: cyclosporine A (calcineurin inhibitor), mycophenolate mofetil (mTOR inhibitor), rapamycin (monophosphate dehydrogenase inhibitor), prednisone (glucocorticoid), and dexamethasone (glucocorticoid).

MSCs can significantly influence T cell differentiation, functions, and survival (Mohammadzadeh et al., 2014; Normanton et al., 2014; Weiss and Dahlke, 2019). Moreover, MSCs can alter the effects of immunosuppressants on T cell proliferation (Hoogduijn et al., 2008). Nevertheless, the knowledge about the combined action of MSCs and immunosuppressive drugs on T cell polarization and survival is still limited. For analysis, we used clinically relevant doses of immunosuppressive medications (Fanigliulo et al., 2015). These doses did not affect MSC survival but substantially suppressed T cell proliferation and cytokine production. It has already been described that MSCs inhibit the apoptosis of T cells and support their survival (Normanton et al., 2014). However, our observations showed that MSCs protected T cells from apoptotic cell death even under immunosuppressive conditions. Besides, the expression of the activation marker CD25 was decreased on T cells. Although the

action of MSCs on T cells under the condition of immunosuppressive agents prevented T cell apoptosis, MSCs reduced the proportion of T cells with activated phenotype.

We also analyzed the effect of MSCs in combination with immunosuppressive drugs on the proportion of Th1/Th2/Th17 cells and Tregs and their lineage-specific cytokine production. The results varied according to the type of immunosuppressive drugs. Most of the used immunosuppressants decreased the proportion of $CD4^+ROR\gamma t^+$ cells, and concurrently the number of $CD4^+IL\text{-}17^+$ cells was also reduced. Nevertheless, in this case, glucocorticoids increased the percentage of $CD4^+ROR\gamma t^+$ cells, which was consistent with the observation of patients suffering from SLE who also elevated the proportion of Th17 cells after glucocorticoid treatment (Prado et al., 2011a). Furthermore, the results revealed that low doses of mycophenolate mofetil also increased the percentage of Th17 cells. It contrasted with studies that described mycophenolate mofetil as an effective inhibitor of Th17 development (Abadja et al., 2011; Eggenhofer et al., 2011). The difference in Th17 development might be elucidated by different metabolic dynamics of mycophenolate mofetil *in vitro* and *in vivo*. However, this unfavorable increase in Th17 cell response was significantly limited in cultures with MSCs.

Similar to the inhibition of Th17 response, we demonstrated that MSCs effectively decreased the proportion of $CD4^+T\text{-}bet^+$ Th1 cells under immunosuppressive drug conditions. MSCs were also involved in the decline of the percentage of $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ cells, especially in the presence of cyclosporine A and dexamethasone. Moreover, combined treatment further limited the expression of GATA-3 and IL-4, characteristics of the Th2 response. Another important subpopulation of T cells is Tregs which have the capacity to suppress immune reactions. Unlike Th17, Th1, and Th2 subpopulations, combined treatment with MSCs and immunosuppressants enhanced Treg differentiation. We observed an increased proportion of $CD4^+IL\text{-}10^+$ cells and enhanced expression of the FoxP3 transcription factor in T cell cultures, especially in the presence of cyclosporine A, rapamycin, and dexamethasone.

Nevertheless, the production of lineage-specific cytokines (IL-17, IL-4, IFN-γ, and IL-10) did not directly correspond to the changes in the expression of lineage-specific transcription factors. A similar result has been described for Tregs in dexamethasone-treated SLE patients who had increased expression of FoxP3 by Tregs, but this expression was not associated with Treg immunoregulatory function (Prado et al., 2011b). These differences may be caused by the ability of immunosuppressive agents to suppress T cell proliferation but not to interfere with T cell activity simultaneously (Miroux et al., 2012).

The therapy combining the administration of MSCs and immunosuppressive drugs represents a promising strategy to reduce immunosuppressive drug doses and attenuate their

adverse effects on body physiology (Hajkova et al., 2017a; Javorkova et al., 2018). However, the interactions of MSCs, immunosuppressive drugs, and the immune system are complex and need subsequent and detailed research.

As mentioned above, another vital cell population participating in immunoregulation is Bregs (Mizoguchi et al., 2002). Due to MSC immunoregulatory properties, by which they can modulate the activity and functions of T cells (Duffy et al., 2011; Hajkova et al., 2017a), we tested the possibility of Breg induction using MSCs in the second part of this work. B cells need to receive the stimulus through TLR to trigger regulatory phenotype development (Fillatreau et al., 2002; Lampropoulou et al., 2008; Yanaba et al., 2008); hence we co-cultivated B cells and MSCs simultaneously in the presence of LPS. Nevertheless, MSCs did not alter the IL-10 production of B cells.

It was also described that the pro-inflammatory cytokines have been important for Breg development and expansion (Holan et al., 2014; Mohd Jaya et al., 2019; Yoshizaki et al., 2012). Therefore, we tested the panel of the cytokines and found that co-cultivation of MSCs and LPS-stimulated B cells in the presence of IFN-γ and IL-4 led to the decrease of IL-10 production by B cells. We further studied these effects on the development of IL-10-producing Bregs. We demonstrated that IFN-γ-primed MSCs decreased the capacity of LPS-stimulated B cells to differentiate into IL-10-producing Bregs via cell-to-cell dependent contact. This suppression involved the COX-2/PGE_2 signaling pathway in MSCs. The MSC effect was reversed using transwell cell-culture techniques or using indomethacin, a COX-2 inhibitor (Hermankova et al., 2016).

Further, we investigated the role of the COX-2 signaling axis on IL-4-primed MSCs, leading to the inhibition of IL-10 production by B cells. The results showed that the suppression was independent of the COX-2 and its products, although the inhibition of IL-10 production by LPS-stimulated B cells co-cultivated with MSCs and IL-4 also required cell contact between MSCs and B cells. We assumed that the soluble PGE_2 molecules, the product of the COX-2, were necessary in very high doses to affect LPS-stimulated B cells; therefore, the cell-to-cell contact was essential. This aspect of PGE_2 action also appeared at T cells. MSCs could alter T cell differentiation via the COX-2/PGE_2 pathway, and cell contact was required as well (Duffy et al., 2011).

Additionally, we examined if MSCs preincubated with IFN-γ or IL-4 were sufficient to suppress IL-10 production by activated B cells. The results revealed that there was a sharp contrast between IFN-γ and IL-4. MSCs only pretreated by IFN-γ also decreased the IL-10 production by B cells but not MSCs in the case of IL-4. MSCs preincubated with IL-4 did not

affect the IL-10 production of LPS-stimulated B cells. For the reduction of IL-10 production, there was necessary simultaneous presence of IL-4 and MSCs.

The observations highlighted the complexity of MSC immunoregulatory properties. It was demonstrated that MSCs through the COX-2/PGE_2 signaling pathway could decrease the pro-inflammatory immune response. By this signaling cascade, MSCs reduced the differentiation of Th17 cells and impaired the activity of $CD8^+$ T cells or NK cells (Duffy et al., 2011; Galland et al., 2017; Li et al., 2014). On the other hand, MSCs supported the induction of Tregs via PGE_2 (English et al., 2009). However, it has been shown recently that human MSCs can induce $CD23^+CD43^+$ IL-10-producing B cells, which effectively alleviated murine colitis. MSCs also interact with Bregs by COX-2/PGE_2 signaling pathway in cell-to-cell contact-dependent manner (Chen et al., 2019). It has also been described that even recombinant PGE_2 could drive and promote the Breg differentiation in a mouse model of viral myocarditis (Chen et al., 2020b), which highlights the plasticity of MSCs in dependence on the local environment.

In the next part of the presented work, we followed up the studies that demonstrated the cytokine milieu plays a role in the induction of Breg differentiation (Holan et al., 2014; Mohd Jaya et al., 2019; Yoshizaki et al., 2012). It has also been described that Bregs could express transcription factor FoxP3, which is characteristic and defining for Tregs (Fontenot et al., 2003; Noh et al., 2012; Park et al., 2016; Vadasz et al., 2015; Vadasz and Toubi, 2017). We chose IFN-γ, IL-4, and TGF-β for the stimulation of mitogen-stimulated lymphocytes to study the activation of suppressive effects of B and T cells and to describe their characteristics. IFN-γ has immunomodulatory properties on activating suppressive functions of LPS-stimulated B cells (Holan et al., 2014). Signals provided by IL-4 are crucial for developing GATA-3^+ Th2 cells, which can further secrete IL-10 (Zheng and Flavell, 1997). Finally, TGF-β is a pivotal factor in developing $FoxP3^+$ Tregs and their regulatory functions (Konkel et al., 2017; Vasileiadis et al., 2018).

The results revealed that the tested cytokines had different effects on IL-10 production by mitogen-stimulated B and T cells. IFN-γ enhanced *Il-10* gene expression, increased the ratio of $IL\text{-}10^+CD19^+$ cells, and consequently supported the secreted amount of IL-10 by stimulated B cells. On the contrary, IL-4 and TGF-β rather decreased the expression of IL-10 in activated B cells on all levels. These cytokines also had the opposite effects on ConA-stimulated T cells. IFN-γ did not affect the IL-10 production by T cells. Otherwise, IL-4 and TGF-β increased the proportion of $IL\text{-}10^+CD3^+$ cells. However, TGF-β did not enhance the concentration of IL-10 in a culture of ConA-stimulated T cells. The results showed that TGF-β also increased the

expression level of the IL-10 receptor on T cells which could lead to subsequent absorption of IL-10.

We further analyzed the role of T cell transcription factors in IL-10 production by mitogen-stimulated B and T cells. The observations demonstrated that B cells did not express GATA-3 and FoxP3, T cell lineage-specific transcription factors, although $FoxP3^+$ Bregs have been described (Noh et al., 2012; Park et al., 2016; Vadasz et al., 2015; Vadasz and Toubi, 2017). The expression of these factors was associated with IL-10 production by ConA-stimulated T cells. As was described, HIF-1α can represent a critical transcription factor for Bregs (Meng et al., 2018). Thus, we tested its expression in mitogen-stimulated lymphocytes. The expression of IL-10 correlated with the expression of transcription factor HIF-1α in LPS-stimulated B cells under cytokine conditions. Nevertheless, ConA-stimulated T cells also expressed HIF-1α, but the selected cytokines did not affect HIF-1α expression in T cells.

To sum up, the results determined that antagonistic cytokines IFN-γ, IL-4, and TGF-β for lineage-specific T cell development modulated IL-10 expression in mitogen-stimulated B and T cells in different ways. Moreover, the selected cytokines regulated IL-10 production by Bregs independently of GATA-3 and FoxP3 transcription factors, which was in a reverse manner than GATA-3-dependent IL-10 production by Th2 cells and the expression of FoxP3 in IL-10-producing Tregs.

Finally, we evaluated the discrepancies between our observations that Bregs do not express transcription factor FoxP3 (Bohacova et al., 2020) and the several studies describing $FoxP3^+$ Bregs (Noh et al., 2012; Park et al., 2016; Vadasz et al., 2015; Vadasz and Toubi, 2017). The authors in these studies used R-PE-conjugated anti-mouse/human FoxP3 antibodies for flow cytometry analysis. On the contrary, we used the antibodies conjugated with AlexaFluor 488 fluorescent dye because R-PE-conjugated antibodies exhibited nonspecific binding to activated B cells in our hands. Thus, we examined the nonspecific binding of R-PE-conjugated antibodies to intracellular markers of stimulated B cells compared to T cells.

In addition to anti-mouse GATA-3 and FoxP3 antibodies conjugated with different fluorescent dyes, we tested a panel of rat and mouse IgG2bκ isotype control antibodies conjugated with other fluorescent dyes purchased from distinct manufacturers. Moreover, we also used various fixation/permeabilization kits to measure intracellular markers of the cells. The mitogen-stimulated B and T cells were utilized the standard protocol for staining of intracellular molecules. Accordingly, the cells were fixed, permeabilized, and stained using isotype control antibodies conjugated with different fluorochromes and measured by a flow cytometer. Up to 30% of LPS-stimulated B cells were R-PE positive if R-PE-conjugated

antibodies were used for staining independently of their specificity. Otherwise, ConA-stimulated T cells did not bind isotype control antibodies. Moreover, the nonspecific binding of R-PE-conjugated antibodies to B cells was independent of mouse strain haplotype.

We also tested the staining of unconjugated R-PE molecules to confirm that the nonspecific binding of antibodies was caused by R-PE molecules. LPS-stimulated B cells bond the R-PE molecules themselves, and it was in a dose-dependent manner. R-PE molecules slightly bond to ConA-stimulated T cells only in the highest concentrations of R-PE. Additionally, we showed that R-PE-conjugated antibodies were bond to cytoplasm markers using confocal microscopy. This nonspecific binding of R-PE-conjugated antibodies appeared only in the case of fixed and stimulated B cells. Unstimulated but fixed B cells or stimulated nonfixed B cells did not nonspecifically bind the R-PE.

Together, results revealed the necessity to verify the specificity of antibodies or probes used for measurements according to the specific cell subpopulation of interest. LPS-stimulated B cells did not express FoxP3 or GATA-3 transcription factors using AlexaFluor 488-conjugated anti-FoxP3 or anti-GATA-3 antibodies for staining, as we have described before (Bohacova et al., 2020). Other authors have suggested that B cells expressed the FoxP3 transcription factor (Noh et al., 2012; Park et al., 2016; Vadasz et al., 2015; Vadasz and Toubi, 2017). The difference between our study and the studies suggesting the existence of $FoxP3^+$ Bregs was the usage of AlexaFluor 488-conjugated anti-FoxP3 and anti-GATA-3 antibodies in our study, while R-PE-conjugated antibodies were used for flow cytometry measurements in all other studies. Only Park et al. (2016) used additional methods, not only fluorescence-based techniques, to prove FoxP3 expression in B cells. Nevertheless, the authors isolated $CD19^+$ cells using magnetic-activated cell sorting columns. However, this kind of separation results in a maximum of 90% purity of B cells, based on our experience. Unfortunately, methods such as western blotting or real-time-polymerase chain reaction do not allow to exclude the eventuality of T cell contamination before B cell analysis, so it might lead to false-positive results.

In summary, the existence of $FoxP3^+$ Bregs requires further research because of discrepancies between studies based on differences in used antibodies. As we have demonstrated, R-PE-conjugated antibodies could provide false-positive results by nonspecific binding to stimulated B cells, in contrast to stimulated T cells. The data based on the usage of R-PE-conjugated antibodies for intracellular marker staining of activated B cells must be taken with precaution.

6. Conclusions

The presented work is based on four original articles: two first author and two co-author ones. The results can be summarized as follows:

I. None of the tested immunosuppressive drugs had a significant effect on the expression of MSC characteristic markers. Nevertheless, the highest concentration of mycophenolate mofetil and rapamycin decreased MSC viability. MSCs reduced apoptosis of T cells caused by the immunosuppressants, but simultaneously MSC protective action did not interfere with reduced T cell activation mediated by these drugs. The combination of MSCs and immunosuppressants significantly decreased the proportion of Th1, Th2, and Th17 cells and their functions. Oppositely, MSCs promoted an anti-inflammatory response based on Tregs in the presence of immunosuppressants.

II. IFN-γ- and IL-4-primed MSCs inhibited the IL-10 production by LPS-stimulated B cells. MSCs preincubated or incubated with IFN-γ suppressed the IL-10 secretion by stimulated B cells. This inhibition was dependent on the COX-2/PGE_2 axis and on cell-to-cell contact. IL-4 also synergized with MSCs to the suppression of IL-10 production by LPS-stimulated B cells. This suppression was independent of the COX-2/PGE_2 signaling pathway but also required cell-to-cell contact. Preincubation of MSCs with IL-4 was not sufficient for the inhibition of IL-10 secretion.

III. Cytokines affecting T subpopulation development also modulate IL-10 expression in LPS-stimulated B cells. IFN-γ significantly enhanced the proportion of IL-10-producing B cells, but IL-4 (Th2 development) and TGF-β (Treg development) decreased this proportion, though they increased the percentage of IL-10-producing T cells. Moreover, the IL-10 expression in LPS-stimulated B cells was independent of GATA-3 and FoxP3 expression, but the expression of IL-10 correlated with the expression of transcription factor HIF-1α. It is in sharp contrast with findings in T cells where IL-10 expression was associated with GATA-3 or FoxP3 expression but without connection to HIF-1α expression in stimulated T cells.

IV. LPS-stimulated B cells, in contrast to ConA-stimulated T cells, nonspecifically bond R-PE-conjugated antibodies used for staining intracellular markers independently of their specificity. The nonspecific binding did not depend on mouse strain haplotype. The activated B cells interacted even with unconjugated R-PE molecules directly and in a dose-dependent manner after fixation.

7. References

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